File Copy

(19) Europäisch s Patentamt
European Patent Office
Office eur péen d s brevets

(11) Publication number: **0 440 304 A1**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: 91200191.4

(22) Date of filing: 30.01.91

(51) Int. Cl.[5]: C12N 15/56, C12N 15/82, A01H 5/00, C12N 1/21, A01N 65/00

(30) Priority: 30.01.90 NL 9000222

(43) Date of publication of application: 07.08.91 Bulletin 91/32

(84) Designated Contracting States: AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: MOGEN INTERNATIONAL N.V.
Einsteinweg 97
NL-2333 CB Leiden(NL)

Applicant: Rijksuniversiteit te Leiden
Stationsweg 46
NL-2312 AV Leiden(NL)

(72) Inventor: Cornelissen, Bernardus Johannes Clemens
Herenweg 29
NL-2361 EB Warmond(NL)
Inventor: Melchers, Leo Sjoerd
Van Swietenstraat 47
NL-2334 EA Leiden(NL)
Inventor: Meulenhoff, Elisabeth Josine Sophie
Orteliuskade 31-I
NL-1507 AE Amsterdam(NL)
Inventor: Van Roekel, Jeroen Sebastiaan Charles
Parnassusweg 35-IV
NL-1077 DD Amsterdam(NL)
Inventor: Sela-Buurlage, Marianne Beatrix
Edisonstraat 58
NL-3817 VP Amerfoort(NL)
Inventor: Vloemans, Alexandra Aleida
Louise Wentstraat 38
NL-2331 NM Leiden(NL)
Inventor: Woloshuk, Charles Peter
Buntgras 19
NL-2318 TA Leiden(NL)
Inventor: Bol, John Ferdinand
Langevoort 34
NL-2341 KA Oegstgeest(NL)
Inventor: Linthorst, Hubertus Josephus Maria
Ank van der Moerstraat 70
NL-2331 HS Leiden(NL)

(74) Representative: Huygens, Arthur Victor, Dr. et al
c/o Gist-Brocades N.V. Patent & Trademarks
Department Wateringseweg 1 PO Box 1
NL-2600 MA Delft(NL)

(54) Fungal resistant plants, process for obtaining fungal resistant plants and recombinant polynucleotides for use therein.

(57) Plants are provided with improved resistance against pathogenic fungi. They are genetically transformed with one or more polynucleotides which essentially comprise one or more genes encoding plant chitinases and β-1,3-glucanases. Preferred are the intracellular forms of the said hydrolytic enzymes, especially preferred are those forms which are targeted to the apoplastic space of the plant by virtue of the modification of the genes encoding the said enzymes. Particularly preferred are plants exhibiting a relative overexpression of at least one gene encoding a chitinase and one gene encoding a β-1,3-glucanase.

EP 0 440 304 A1

FUNGAL RESISTANT PLANTS, PROCESS FOR OBTAINING FUNGAL RESISTANT PLANTS AND RECOMBINANT POLYNUCLEOTIDES FOR USE THEREIN

FIELD OF THE INVENTION

The invention lies in the area of recombinant DNA technology, especially in conjunction with the genetic manipulation of plants and concerns a process for obtaining fungal resistant plants due to genetic manipulation, as well as genetically manipulated plants and plant cells themselves (including subparts of the genetically manipulated plants as well as progeny obtained by asexual or sexual propagation) and recombinant polynucleotides (DNA or RNA) which can be used for the genetic manipulation.

BACKGROUND

Most agricultural and horticultural crops are under a constant threat due to fungal attack. To protect the crops from significant losses due to fungal disease, the crops and sometimes the soil in which the crops are grown are periodically treated with large amounts of fungicides. These fungicides form a heavy burden on costs of crop growing, and more importantly on the environment and the growers. Moreover the treatment is very labour intensive. Therefore, there is a need for less costly and safer methods to protect plants from fungal attack which, preferably, are devoid of the need of repeated human involvement.

Induced resistance

In plants generally several types of resistance against pathogens occur; non-host resistance, "horizontal" or partial resistance and "vertical" resistance. None of these forms of resistance is particularly well understood in molecular terms. In addition to these constitutively expressed forms of resistance there is a resistance mechanism that can be induced by certain pathogenic infections as well as by a number of biotic and abiotic factors. This induced resistance is very broad and is directed against various pathogens, including fungi. This is further illustrated below.

Inoculation of the lower leaves of a hypersensitively reacting tobacco cultivar (Nicotiana tabacum cv Samsun NN) with tobacco mosaic virus (TMV) results in the formation of local lesions on the inoculated leaves. The non-inoculated leaves appear resistant to a second infection with TMV after 3 days; this resistance lasts at least twenty days, and an optimal resistance is obtained after 7 days. The resistance against the second infection is also directed to other viruses, such as tobacco necrosis virus, tobacco ringspot virus (Ross & Bozarth, 1960; Ross, 1961), and fungi, such as Thielaviopsis basicola (Hecht & Bateman, 1964), Phytophthora nicotianae and Peronospora tabacina (McIntyre & Dodds, 1979; McIntyre et al., 1981).

The phenomenon of induced resistance has been studied in numerous other host plants and in combination with several other pathogens as well (Kuc, 1982; Sequeira, 1983). The general picture emerging from these studies is that a hypersensitive response is accompanied by resistance against a broad range of pathogens, irrespective of the type of pathogen having caused the first infection.

Proteins expressed concomitant with induced resistance

Together with the resistance a great number of proteins is synthesized, which are absent before infection.

Roughly three categories of proteins can be discerned:

1) Key-enzymes in the synthesis of secondary metabolites, such as phytoalexins, which exhibit an antimicrobial effect, and precursors of lignin; the latter is used in the reinforcement of plant cell walls after pathogen invasion. These enzymes, or their messenger RNAs are mainly found in cells in the immediate vicinity of the site of infection (Elliston et al., 1976; Cramer et al., 1985; Bell et al., 1986).

2) Hydroxyproline rich glycoproteins (HRGPs) or extensins, which can be incorporated into the cell wall and possibly function there as a matrix for the attachment of aromatic compounds like lignin (Fry, 1986). HRGPs are important structural components of plant cell walls, and their accumulation occurs in reaction to fungi, bacteria and viruses (Mazau & Esquerré-Tugayé, 1986). In contrast to the situation with the key-enzymes mentioned above, HRGPs and their mRNAs are found in substantial amounts in non-infected parts of th plant as well as around the sit of infection (Showalter et al., 1985).

3) A third group of induced genes ncodes proteins which accumulate both inside th cells and in the

apoplastic space. Among these proteins are hydrolytic enzymes such as chitinases and glucanas s. After a necrotic infection these enzymes can often be found throughout the plant, including the non-infected parts, in higher concentrations than before infection. Increased synth sis f these nzymes appears to be induced also by microbial licitors, usually fungal cell wall preparations (Darvill & Albersheim, 1984; Toppan & Esquerré-Tugayé, 1984; Mauch et al., 1984; Chappel et al., 1984; Kombrink & Hahlbrock, 1986; Hedrick et al., 1988).

Structure of fungal cell walls

The cell walls of fungi are known to consist of a number of different carbohydrate polymers. Most fungi, with the exception of the Oomycetes, contain considerable amounts of chitin. Chitin is a polymer of N-acetyl glucosamine molecules which are coupled via β-1,4 linkages and, in fungal cell walls, are often associated with β-1,3/β-1,6 glucan, polymers of glucose with β-1,3 and β-1,6 linkages. Fungi from the group of Zygomycetes do not contain glucans with β-1,3 and β-1,6 linkages, while in most of the Oomycetes the glucans are associated with cellulose (for an overview, vide: Wessels and Sietsma, 1981).

In vitro degradation of isolated fungal cell walls

It has been known for a long time that isolated cell walls of fungi can be degraded in vitro by plant extracts (Hilborn & Farr, 1959; Wargo, 1975; Young & Pegg, 1982) and also by chitinase and β-1,3-glucanase preparations from microbial origin (Skujins et al., 1965; Hunsley & Burnett, 1970; Jones et al., 1974).

More recently a purified endo-β-1,3-glucanase from tomato in combination with an exo-β-1,3-glucanase of fungal origin were shown to be capable of hydrolysing isolated cell walls of the fungus Verticillium albo-atrum. Each of the preparations separately did not have activity (Young & Pegg, 1982). A purified β-1,3-glucanase from soybean (Keen & Yoshikawa, 1983), as well as a purified chitinase from bean (Boller et al., 1983) have also been shown to be capable of degrading isolated cell walls of fungi in vitro. When pea chitinase and β-1,3-glucanase were tested on isolated cell walls of Fusarium solani, both appeared to be active; in combination they appeared to work synergistically (Mauch et al., 1988b).

It is not known whether these hydrolytic enzymes can degrade the polymer compounds in cell walls of living fungi effectively, if at all.

Inhibition of fungal growth on synthetic media by chitinases and glucanases from plant origin

Some chitinases and glucanases of plant origin are capable of inhibiting the growth of fungi on synthetic media. Chitinase purified from bean is capable of inhibiting the growth of the fungus Trichoderma viride in agar plate assays (Schlumbaum et al., 1986). A combination of chitinase and β-1,3-glucanase, both purified from pea pods, do inhibit the growth of some fungi on agar plates, whereas other fungi are not inhibited. The Ascomycete Cladosporium cucumerinum appeared slightly sensitive, while the Oomycetes Phythophthora cactorum, Pythium aphanidermatum and Pythium ultimum are insensitive.

Pea chitinase alone has effect on the growth of T.viride, while β-1,3-glucanase inhibits the growth of Fusarium f.sp. pisi. It was established that in these assays the inhibition of fungal growth was due to lysis of the hyphal tips (Mauch et al., 1988b). Apparently the hydrolytic enzymes do have access to their substrate in the cell walls of living fungi, when grown on synthetic media, although at least some of the active plant hydrolytic enzymes seem to be specific to certain fungi.

Little is known about the effect of hydrolytic enzymes on fungi in the biotrope, i.e. in the soil or on plant leaves, and although some of these enzymes are putative candidates for a role in fungal resistance, evidently, not all chitinases and glucanases have activity against living fungi. Possibly, the stage and site of infection at which hydrolytic enzymes come into contact with the invading fungus may be of great importance.

Occurrence of chitinases and glucanases in plants

As far as known, chitinases and β-1,3-glucanases occur in most if not all plant speci s, both in monocotyledonous and dicotyledonous plants. At least two classes of chitinases and two classes of glucanases can b discerned: intracellular and extracellular. Both chitinase and glucanase genes of one particular class appear to be encoded by gene families.

Natural expression of chitinase genes and glucanase genes in plants

Chitinase and glucanase gen s are known to be expressed in plants both constitutively and in a strictly regulated fashi n.

Chitinases and β-1,3-glucanases are constitutively synthesised in roots of tobacco plants (Felix and Meins, 1986, Shinshi et al., 1987,; Memelink et al., 1987, 1989). Nevertheless tobacco plants are not resistant to infection of Phytophthora parasitica var. nicotianae (a root pathogen of tobacco). However, resistance against this pathogen can be induced in tobacco plants, following inoculation with TMV (McIntyre & Dodds, 1979). This suggests that a complex of yet unknown factors other than, or in addition to, chitinases and glucanases, may be involved in fungal resistance.

On the other hand, plant species are known which seem to be resistant to fungal infection, although no significant increase in the levels of chitinases or glucanases can be observed. For instance, in tomato a compatible interaction with the fungus Phytophthora infestans causes a systemic resistance (Christ & Mösinger, 1989), i.e. a resistance to infection throughout the whole plant, although chitinases or glucanases cannot be detected in such leaves (Fischer et al., 1989). Apparently there is no clear correlation between expression of the genes encoding hydrolytic enzymes and fungal resistance.

In addition to these observations, some chitinases exhibit a regulated expression pattern which does not immediately suggest a correlation with fungal resistance.

For example, genes encoding chitinases are known to be expressed in a developmentally regulated manner in, inter alia, tobacco flowers (Lotan et al., 1989). Glucanases are known to occur in large quantities in seedlings of barley (Swegle et al., 1989; Woodward & Fincher, 1982; Hoj et al., 1988, 1989).

In tobacco cell suspensions the synthesis of intracellular chitinases and glucanases can be inhibited by the addition of cytokinins or auxins (Mohnen et al., 1985; Felix & Meins, 1986; Shinshi et al., 1987; Bauw et al., 1987).

The synthesis of the same hydrolytic enzymes can be induced by cytokinin when this hormone is added to the growth medium in which normal tobacco plants are grown axenically. Under certain circumstances the plant hormone ethylene can also induce the synthesis of chitinase and glucanase (Felix & Meins, 1987).

In the roots and lower leaves of both soil-grown and axenically grown tobacco plants, intracellular chitinases and glucanases can be detected, while in upper leaves they can not be detected at all, or to a much lesser extent (Felix & Meins, 1986; Shinshi et al., 1987; Memelink 1987, 1989). Thus, there is also organ-specific expression of the intracellular chitinases and glucanases.

The regulation of expression of the genes coding for extracellular chitinases and glucanases is hardly, or not at all, influenced by cytokinin (Memelink et al 1987., 1989). In tobacco flowers the extracellular chitinases are expressed specifically in anthers, sepals and the ovary.

Thus, there is at least an organ-specific expression of the genes coding for the extracellular chitinases as well.

Fungal resistant plants expressing chimeric chitinase genes

Notwithstanding the many still unelucidated features concerning the nature and the role of hydrolytic enzymes in fungal resistance, some initial successes have been reported in providing plants with diminished sensitivity to fungal attack.

In US Patent 4,940,840, tobacco plants expressing a bacterial chitinase gene (i.e. the chiA gene from Serratia marcescens) have been shown to be less sensitive to the fungus Alternaria longipes.

In the International Patent Application WO 9007001 the plant species tobacco and canola, expressing a bean chitinase under regulation of a strong viral promoter or a plant promoter, appear to be less sensitive to two of the tested fungi, namely Botrytis cinerea and Rhizoctonia solani.

It is not known, however, whether these plants are effectively resistant to other fungi as well.

In European Patent Application EP-A-0 292 435 it was suggested that resistance to certain classes of fungi may be conferred by the introduction of a gene that expresses chitinase in the plant tissues.

Mention was made of a preference in certain cases to target gene products into the mitochondria, the vacuoles, into the endoplasmic vesicles or other cell parts or even into the intercellular (apoplastic) spaces.

There was no teaching of the type of chitinase or of the preferred site of action of the chitinase, in order to obtain the desired effect.

EP-A-0 270 248 proposes a mechanism to target a bacterial gene (the β-glucuronidase gene from E.coli) to the plant cell wall by using the leader sequence of the polygalacturonase g ne from tomato. It was, inter alia, proposed to target chitinases or glucanases to the plant cell wall to combat fungal attack.

Results were not shown, nor was indicated which hydrolytic nzym s sh uld be used, or how intracellular plant proteins must be targeted outside the plant cell.

In EP-A-0 332 104 genetic constructs are described comprising chemically regulative sequences derived from plant genes, among which the so-called PR-genes, including those coding for chitinase and glucanase. No results of fungal resistant plants were shown.

Summary of the state of the art

Plants contain at least two classes of chitinases and β-1,3-glucanases: extracellular and intracellular. The expression of the genes encoding the said hydrolytic enzymes is not constitutive, at least not in all tissues, but is among other things regulated in a developmental or tissue-specific fashion. However, the expression of the genes can also be induced under certain stress-conditions, such as an infection by a necrotisizing pathogen. In most cases, induction of the synthesis of chitinases and β-1,3-glucanases is accompanied by the induction of resistance against a broad range of pathogens, including phytopathogenic fungi. Whether there is a causal relation between fungal resistance and expression of the genes encoding hydrolytic enzymes is not clear.

Cell walls of phytopathogenic fungi contain glucans and often a certain amount of chitin. These carbohydrate polymers are substrates for glucanases and chitinases, respectively. It is attractive to hypothesize that both hydrolytic enzymes are responsible for the observed resistance. However, this is far from obvious, in view of many observations which are clearly in conflict with this hypothesis.

Hence, it is still unclear whether hydrolytic enzymes have a significant role in fungal resistance, or, when they appear to have so, how substantial their role in fungal resistance is. It seems at least doubtful that any chitinase can confer broad range protection of plants against phytopathogenic fungi. Generally, it is even questionable if chitinases and glucanases by themselves are capable of providing sufficient protection against a broad range of plant pathogenic fungi.

There is still little basic understanding of the role of hydrolytic enzymes in the complex process of acquiring (induced) fungal resistance. However, there is a need for a method to effectively protect plants against (a broad range of) phytopathogenic fungi, by means of genetic modification.

SUMMARY OF THE INVENTION

The aim of the present invention is to provide plants which have improved resistance to fungal attack. Thereto, plants are genetically transformed by introducing into the genome of the said plants at least one recombinant polynucleotides comprising one or more genes encoding an intracellular chitinase of plant origin, under the control of a promoter which is not naturally associated with that gene.

More in particular the invention provides plants having improved resistance to fungal attack, by virtue of the expression of at least one recombinant DNA-construct that comprises a DNA-sequence, encoding at least one intracellular plant chitinase, which is modified such that the intracellular chitinase becomes secreted into the apoplastic space.

In a preferred embodiment, the invention provides plants exhibiting a more effective protection against fungal attack due to the expression of a gene encoding a chitinase, preferably an intracellular chitinase, and a gene encoding a glucanase, under the control of a promoter that allows suitably strong expression, in one or more tissues.

In a still further preferred embodiment, the invention provides plants constitutively expressing a gene encoding an intracellular chitinase of plant origin which is targeted to the apoplastic space and, additionally, one or more genes encoding a hydrolytic enzyme from the group consisting of intracellular chitinases, extracellular chitinases, intracellular glucanases and extracellular glucanases.

One especially preferred embodiment is a plant expressing the genes encoding an intracellular chitinase, an extracellular chitinase, an intracellular β-1,3-glucanase, and an extracellular β-1,3-glucanase. Of these, genes encoding the intracellular forms of the mentioned plant hydrolytic enzymes are particularly preferred. Still more preferred is the use of the genes encoding intracellular hydrolytic enzymes, modified by genetic manipulation as to provide for apoplast targeting. In order to achieve apoplast-targeting of the intracellular hydrolytic enzymes, the 3'-end of the gene encoding the C-terminal end of the intracellular hydrolytic enzymes is modified in order to establish, upon expression of the genes, the absence of the C-terminal amino acids that are involv d in intracellular targeting of th respective enzymes. Generally such modification results in the absence of at least 3 amino acids of the C-terminal end, or as many amino acids as desired, as long as the enzymatic function and/or other relevant domains of the protein are not negatively affected. Preferably such modification results in the deletion of between 3 and 25 amino acids in

the case of intracellular β-1,3-glucanases, and between 3 and 10 amino acids in the case of intracellular chitinases. More preferred are deletions of 4-8 amino acids.

Further embodiments of th invention are the recombinant DNA molecules, comprising one or more plant expressible DNA sequences encoding at least one intracellular chitinase of plant origin which is modified to achieve targeting of the chitinas to the apoplastic space, and, if desired additional DNA sequences encoding one or more hydrolytic enzymes selected from the group consisting of extracellular chitinases, intracellular glucanases and extracellular glucanases.

Certain preferred embodiments are the intracellular chitinase genes located on the EcoRI-SstI fragment of pMOG200; the extracellular chitinase gene from petunia hybrida, located on pMOG200; the intracellular β-1,3-glucanase gene located on the XbaI-SstI fragment of pMOG212; the gene encoding the extracellular β-1,3-glucanase which is located on the SstI-HindIII fragment of pMOG212, or genes which are essentially homologous to the said genes.

Especially preferred are modified versions of the genes encoding intracellular forms of the said hydrolytic enzymes, which provide for apoplast-targeting. This includes the modified intracellular chitinase gene of pMOG189 (or truncated forms thereof which retain antifungal activity), as well as modified forms of intracellular chitinase genes, which are essentially homologous to the intracellular chitinase gene of pMOG189. Also preferred is the modified intracellular glucanase gene of pMOG512, in which a stopcodon is introduced into the coding region to provide for apoplast-targeting of the produced intracellular β-1,3-glucanase.

Also provided are cloning vectors, expression vectors and transformation vectors containing DNA-sequences comprising the said genes, as well as microorganisms containing said DNA-sequences.

Of these vectors the plasmids pMOG200 and pMOG212, and derivatives thereof are preferred.

Further embodiments of the present invention include whole fungal resistant plants obtained by the processes according to the said invention, protoplasts, cells, parts (such as seeds, fruits, leafs, flowers, and the like), and any other part of the plant that can be reproduced either sexually, asexually or both, and progeny of the said plants.

The advantages and the field of application will be readily understood from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence and the deduced amino acid sequence of a complete cDNA corresponding to an extracellular chitinase from *Petunia hybrida*. The vertical arrow shows the cleavage site of the signal peptide.

Figure 2 shows the nucleotide sequence and the deduced amino acid sequence of a BamHI DNA fragment corresponding to an intracellular chitinase from tobacco. The sequence of nucleotide 2 through 22 originates from a synthetic fragment, while the nucleotides 23-27 form the remainder of the EcoRI recognition site. The PstI recognition site (5'-CTGCAG-3') is found at position 129-134. The last 21 nucleotides of the sequence succesively represent a filled in EcoRI recognition site, originating from an EcoRI linker-molecule used for the construction of the cDNA library, a SmaI and a BamHI recognition site, both originating from the polylinker of pIC19H. The arrow shows the cleavage site of the signal peptide.

Figure 3 shows the nucleotide sequence and the deduced amino acid sequence of a gene coding for an extracellular β-1,3-glucanase from tobacco. The vertical arrow shows the location in the amino acid sequence where the signal peptide is cleaved. The position of the intron is indicated; the sequence of the intron is only given in part.

Figure 4 shows the nucleotide sequence and the deduced amino acid sequence of a gene coding for an intracellular β-1,3-glucanase from tobacco. The vertical arrow shows the location in the amino acid sequence where the signal peptide is cleaved.

Figure 5 shows a schematic representation of expression vector pMOG181. Amp^r stands for the ampicilline resistance gene. A restriction enzyme recognition site between brackets shows that the concerned site is no longer present in the plasmid.

Figure 6 shows a schematic representation of vector pMOG183, a derivative of pMOG181 wherein the EcoRI recognition site is replaced by a SstI site.

Figure 7 shows a schematic representation of vector pMOG184, a derivativ of pMOG181 wherein the HindIII recognition site is replaced by a SstI sit .

Figure 8 shows a schematic representation of vector pMOG185, a derivative of pMOG184 wherein the EcoRI recognition site is replaced by a XbaI site.

Figure 9 shows a schematic representation of th binary vector pMOG23.

Figur 10 shows a schematic representation of the binary vector pMOG22, a derivative of pMOG23 wherein the kanamycin resistance gen (NPTII) is replaced by a hygromycin resistance g n (HPT).

Figure 11 shows a schematic representation of the plasmid pMOG200, a derivative of pMOG23 wherein two expression cassettes are cloned into the polylinker, viz., one with the coding sequence for an intracellular chitinase (ChiI) and one with the coding sequence for an extracellular chitinase (ChiE). The arrow provides the direction of the transcription in the cassettes, beginning with the CaMV 35S promoter.

Figure 12 shows a schematic representation of plasmid pMOG212, a derivative of pMOG22 wherein two expression cassettes are cloned into the polylinker, viz., one with the coding sequence for an extracellular β-1,3-glucanase (GluE) and one with the coding sequence for an intracellular β-1,3-glucanase (GluI). The arrows give the direction of transcription beginning with CaMV 35S promoter.

DEFINITIONS

For the purpose of the present invention it is understood that an extracellular protein is a protein which, after proper expression in the original plant, is localised in the apoplastic space.

Consequently, an intracellular protein is a protein which, after proper expression in the plant of origin, is localised intracellularly.

The apoplastic space is defined herein as the extracellular space, including the plant cell wall.

For the purpose of this invention a protein is said to be localised intracellularly if it is localised in any compartment of the cell that does not form part of the apoplastic space; these compartments include nuclei, chloroplasts, mitochondria, vacuoles, endoplasmatic reticulum, other membranous organelles, the cytoplasm, and all membranes, including the plasma membrane.

Genes are said to be essentially homologous if their DNA sequences correspond for more than 60%, unless stated otherwise.

With chimeric genes is meant, genes that are physically coupled to regulatory sequences (e.g. promoters, enhancers, or DNA-sequences that impart a specific mode of regulation of the gene), which are not naturally coupled to that gene, in order to alter its authentic mode of regulation of expression.

By plant is meant any monocotyledonous or dicotyledonous plant, including progeny, or parts of such plants, cells or protoplasts, and the like, and any other plant material that is amenable to transformation and subsequent regeneration into a whole plant.

With relative overexpression is meant any expression of a chimeric gene that eventually leads to higher levels of the encoded protein in a preselected plant part, as compared with the expression of the non-chimeric gene in the same plant part in the plant of origin.

DETAILED DESCRIPTION OF THE INVENTION

In the light of their assumed involvement in fungal resistance, it was surprisingly found that purified extracellular chitinases from tobacco and petunia do not have a significant antifungal effect when compared to intracellular chitinases. In an antifungal assay, equal amounts of chitinolytic activity of purified intracellular and extracellular chitinases, rather than equal amounts of protein, were compared. The antifungal activity of the tested extracellular forms was practically undetectable.

Expression of a chimeric gene encoding an extracellular chitinase in a transformed plant as such, is therefore not sufficient to provide for fungal resistance. Nevertheless, it can not be entirely excluded that extracellular chitinases play a supportive role in fungal resistance, by increasing the antifungal effect of other hydrolytic enzymes present. This observation has important implications for the engineering of fungal resistance in plants, based on expression of chimeric genes encoding plant hydrolytic enzymes.

Comparison of the C-terminal ends of several homologous proteins (particularly of chitinases, and glucanases), which differ essentially in their localisation, revealed that intracellular proteins often have an extension of about 3 to 25 (in the case of intracellular β-1,3-glucanases), or 3 to 10 (in the case of intracellular chitinases) amino acid residues compared to their extracellular analogues. It was surprisingly found, that deletion of about 6 amino acid residues at the C-terminal portion of an intracellular tobacco chitinase results in secretion of the protein to the apoplastic space. Apparently the C-terminal extension functions as a 'vacuole-targeting' signal.

We believe this is the first demonstration of apoplast- targeting of chitinases that naturally occur in the vacuole of a plant cell. This finding can be suitably applied for the targeting of vacuolar proteins (e.g. proteins which are localised in the vacuole) to the apoplastic space.

A very effective site of action of hydrolytic enzymes in the protection of transformed plants against a range of plant pathogenic fungi is believed to b the apoplastic space. Hence, to obtain improved fungal

resistance it is advantageous if plants are transformed with a recombinant DNA construct comprising a gene encoding a chitinase (or a truncated form thereof, which comprises the antifungal domains or parts) which exerts its action in the apoplastic space of the plant, either naturally or by virtue of genetic modification.

To obtain such plants, it is preferred that plants are transformed with a recombinant DNA construct comprising a gene encoding an intracellular chitinase, which is modified such that the C-terminal amino acids involved in vacuolar targeting are not present (e.g. by introducing a translational stopcodon in the coding region of the gene, or otherwise), resulting in apoplast-targeting of (most of) the intracellular chitinase produced in that plant.

To evaluate the possibility of targeting intracellular hydrolytic enzymes to the apoplastic space, without a significant adverse effect on the antifungal activity, the following experiment was carried out.

Plants were transformed with DNA constructs essentially comprising the following genes:

1°. a gene encoding a petunia extracellular chitinase,

2°. a gene encoding a tobacco intracellular chitinase,

3°. a gene encoding a tobacco intracellular chitinase, modified as to obtain apoplast-targeting of the chitinase (targeting construct), or

4°. the petunia extracellular chitinase gene, and the modified tobacco intracellular chitinase gene (targeting-construct).

All genes were placed under the control of the cauliflower mosaic virus 35S promoter. Of each category of the transformed plants good expressors of the chimeric genes were selected and subjected to isolation of extracellular fluids (EF) and total protein extracts (TE) of leaves. The antifungal effect of the different fractions from the plants 1 through 4 were determined on the test fungus Fusarium solani. Neither the EF nor the TE of plant 1, expressing the petunia extracellular chitinase had any antifungal activity, as was expected from the experiments using the purified hydrolytic enzymes. The EF of plant 2 had residual antifungal effect (probably due to leakage from the cell of the (relatively over-)expressed intracellular chitinase), whereas the total protein extract showed a strong antifungal effect. Of plant 3, expressing the modified apoplast-targeted intracellular chitinase gene, both the EF and the TE exhibited a strong antifungal effect; this, most importantly, proves that the targeted intracellular chitinase of plant 3 still has antifungal activity. Thus, unexpectedly, the deletion of the C-terminal vacuole targeting signal does not significantly affect the antifungal activity of the chitinase.

Plants may be even more effectively protected against fungal attack if they express both an intracellular chitinase and a modified (apoplast-targeted) intracellular chitinase.

Thus, the invention provides plants having improved fungal resistance, as well as methods to obtain such plants.

In a first aspect of the present invention it has been found that the intracellular forms of tobacco and Petunia chitinases are preferred over extracellular chitinases. Therefore, intracellular chitinases are preferred which are essentially homologous to the intracellular chitinases of tobacco. Preferably this homology of intracellular plant chitinases should be larger than 50% on protein level, more preferably more than 60%, most preferably more than 70%.

A second aspect of the invention is the unexpected finding that the strong antifungal effect of intracellular chitinases is retained after modification of the C-terminal end of the protein. Thus, to improve fungal resistance in transformed plants the most potent hydrolytic enzymes, i.e. the intracellular forms, are selected, and these hydrolytic enzymes, or the truncated forms, which comprise the active antifungal domains/parts, and targeted to the apoplastic space, where their antifungal effect is optimal.

In a following series of experiments the combined effect of chitinases and glucanases in total protein extracts and extracellular fluids of leaves of transgenic plants was investigated.

Tobacco plants were transformed with a recombinant DNA construct essentially comprising:

1°. a gene encoding a tobacco intracellular β-1,3-glucanase, targeted to the apoplast by modification of the C-terminal end;

2°. a gene encoding a tobacco intracellular chitinase, and the tobacco intracellular β-1,3-glucanase, both targeted to the apoplast, by modification of the C-terminal end of the hydrolytic enzymes.

Again, transgenic tobacco plants that were good expressors of the chimeric genes were selected, and subjected to isolation of extracellular fluid (EF) and total protein extract (TE) of leaves. Both the EF and TE of plant 1, expressing the intracellular β-1,3-glucanase that was targeted to the apoplast, exhibited a weak antifungal effect on the fungus Fusarium solani. The EF and TE of plant 2, expressing both the apoplast-targeted intracellular chitinase and th apoplast-target d intracellular β-1,2-glucanase, exhibited a surprisingly strong antifungal effect; this effect was slightly higher than that of the EF and TE of plant 3 of the former experiment (expressing only the gene encoding the apoplast-targeted intracellular chitinase).

It can be concluded from these experiments that modification of the C-terminal end of the intracellular

β-1,3-glucanase succesfully leads to apoplast-targeting of (m st of) the enzyme, and that the C-terminal modification does not adversely affects the antifungal activity of the intracellular β-1,3-glucanas . Moreover, it is shown that the antifungal effect of th xpressi n of both an intracellular chimeric chitinase gene and an intracellular chimeric β-1,3-glucanasegene is larger than the effect of the xpression of each of the genes alone.

Thus, in a third aspect of the invention plants having improved fungal resistance are provided, expressing a chimeric plant chitinase gene and a chimeric plant glucanase gene, both under the regulation of the CaMV 35S promoter.

In a preferred embodiment of the present invention plants are provided which have been transformed with one or more genes encoding intracellular forms of plant hydrolytic enzymes, in a plant expressible form. Especially preferred are plants which express one or more genes encoding intracellular forms of plant hydrolytic enzymes, which by virtue of modification of the C-terminal end are targeted to the apoplast. Still further preferred are plants which are transformed with at least a gene encoding an intracellular chitinase gene and an intracellular β-1,3-glucanase gene. It will be advantageous if these latter plants express the modified forms of the hydrolytic enzymes, to achieve apoplast-targeting of the said enzymes.

Another preferred embodiment of the invention is a plant constitutively expressing an intracellular chitinase, preferably targeted to the apoplast, an extracellular chitinase, an intracellular glucanase, preferably targeted to the apoplast, and an extracellular glucanase.

In principle any combination of genes encoding plant hydrolytic enzymes can be chosen, modified or unmodified, as long as suitably high expression of these genes does not impair cell function of the transformed plant host. In addition to genes encoding plant hydrolytic enzymes, other plant or non-plant genes (e.g. derived from bacteria, yeast, fungi, or other sources) may be used as well.

The plant genes encoding the hydrolytic enzymes may either be endogenous or exogenous to the plant that is to be transformed.

It will be readily understood, that, in addition to the chitinase and β-1,3-glucanase genes mentioned, genes encoding hydrolytic enzymes can be readily isolated from other plant species as well. Moreover, the genes as meant by the present invention may be entirely synthetic.

Genes or cDNAs coding for the desired hydrolytic enzymes can for instance be isolated from tobacco (e.g. Legrand et al., 1987; Shinshi et al., 1987), tomato (Joosten et al., 1989), a basic intracellular chitinase can be isolated from potato (Gaynor, 1988; Kombrink et al., 1988), an extracellular chitinase can be isolated from cucumber (Métraux & Boller, 1986; Métraux et al., 1986), and both intracellular chitinases and glucanases can be isolated from bean (Broglie et al., 1986; Vögeli et al., 1988; Mauch & Staehelin, 1989).

Furthermore, chitinases and β-1,3-glucanases can be isolated from pea, using chitosan as inducing compound (Mauch et al., 1984). Further analysis revealed the presence of at least five hydrolases, viz. two basic β-1,3-glucanases and three basic chitinases (Mauch et al., 1988a). Intracellular and extracellular chitinases which are serologically related to an intracellular chitinase from bean can be isolated from Allium porrum L. (Spanu et al., 1989). Endochitinases and glucanases can also be isolated from maize, following inoculation of leaves with BMV (bromine mosaic virus) (Nasser et al., 1988). Chitinases which are serologically related to an intracellular endochitinase from bean (Swegle et al., 1989) can be isolated from barley (Hordeum vulgare). Also β-1,3-glucanases, as well as other classes of glucanases, can be isolated from barley (Balance et al., 1976; Hoj et al., 1988, 1989). At least 4 different chitinases and 5 different β-1,3-glucanases are known to exist in oat (Fink et al., 1988).

It will be understood that sources for obtaining hydrolytic enzymes for protecting plants against fungal attack, are not limited to the list given above, which is only given as illustration.

cDNAs encoding plant chitinases and β-1,3-glucanases are suitably obtained by immunological screening of a cDNA-expression library, made on polyA^{+}-RNA, isolated from plants after induction of the synthesis of the hydrolytic enzymes, using an antibody against the desired hydrolytic enzyme. In order to be expressed properly the gene must be operably linked to a promoter.

The choice of the promoter is dependent on the desired level of expression and the desired way of regulation of the gene under its control. This is all within ordinary skill.

Preferably strong constitutive promoters are used which function throughout the whole plant, with as little as possible restrictions with respect to developmental patterns. One example of a constitutive promoter for high level expression is the CaMV 35S promoter. This promoter may be flanked by so-called enhancer sequences (McGuilley et al., 1987) to further enhance expression levels. Other examples of high-level, light-inducible, promoters ar , among others, the ribulose bisphosphate carboxylase small subunit (rbcSSU) promoter, the chlorophyl a/b binding protein (Cab) promoter, and the like. Occasionally, it may be desirable to restrict expression of the introduced chimeric genes to one or a f w pre-selected tissues, for instance those that are targets for fungal attack, such as roots and pidermal cells, and the like. A well known

example of a tissue-specific promoter is for example the root-specific patatin class-II promoter. Expression of chimeric genes may be dependent on xogenous stimuli as well, like wounding, drought, temperature, and the like.

Generally the gen (s) of choice is/are contain d in an xpression cass tte, which comprises at least a promoter and a transcription terminator, which may be for ign to the g ne. It is well known how such elements should be linked in order to function properly and this can be determined without practising inventive skill. Occasionally eukaryotic (genomic) genes contain introns. The presence of the latter, either naturally or introduced by genetic modification, is not particularly relevant to the invention. The techniques for gene manipulation are readily available to a person skilled in the art (vide e.g.: Maniatis et al., 1982).

In addition to genes encoding hydrolytic enzymes also genes encoding other proteins having an extra effect on pathogen resistance may be introduced in the plant of interest, in order to improve the effect or broaden pathogen range. Such proteins are suitably chosen from the group consisting of e.g. lectins, cow pea trypsin-inhibitor (CpTI), Bacillus thuringiensis toxins, and the like.

To obtain transgenic plants capable of constitutively expressing more than one chimeric gene, a number of alternatives are available, which are encompassed by the present invention, including the following:

A. the use of one recombinant polynucleotide, e.g a plasmid, with a number of modified genes physically coupled to one selection marker gene.

B. Cross-pollination of transgenic plants which are already capable of expressing one or more chimeric genes coupled to a gene encoding a selection marker, with pollen from a transgenic plant which contains one or more gene constructions coupled to another selection marker. Afterwards the seed, which is obtained by this crossing, is selected on the basis of the presence of the two markers. The plants obtained from the selected seeds can afterwards be used for further crossing.

C. The use of a number of various recombinant polynucleotides, e.g. plasmids, each having one or more chimeric genes and one other selection marker. If the frequency of cotransformation is high, then selection on the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.

D. Consecutive transformations of transgenic plants with new, additional chimeric genes and selection marker genes.

E. Combinations of the above mentioned strategies. The actual strategy is not critical with respect to the described invention and can be easily determined depending on factors such as the desired construct, the materials available and the preference of the skilled workers.

For the transformation of plants several techniques are available. The choice of the technique is generally not critical to the invention, as long as the transforming genetic construct, comprising the genes and regulatory elements according to the invention, can be introduced into a plant and become stably integrated into the genome of that plant.

Some examples for purposes of illustration are transformation of protoplasts using the calcium/polyethylene glycol method (Krens et al., 1982; Negrutiu et al., 1987), electroporation (ref.) and microinjection (Crossway et al., 1986), (coated) particle bombardment (Klein et al., 1987), infection with viruses and the like. After selection and/or screening for the tranformed plant material, the transformed material is regenerated into whole plants, using methods known in the art.

Subsequently transformed plants are evaluated for the presence of the desired properties and/or the extent to which the desired properties are expressed. A first evaluation may include the level of expression of the newly introduced genes, the level of fungal resistance of the transformed plants, stable heritability of the desired properties, field trials and the like.

Secondly, if desirable, the transformed plants can be cross-bred with other varieties, for instance varieties of higher commercial value or varieties in which other desired characteristics have already been introduced, or used for the creation of hybrid seeds, or be subject to another round of transformation and the like.

Plants, or parts thereof of commercial interest, with improved resistance against phytopathogenic fungi can be grown in the field or in greenhouses, and subsequently be used for animal feed, direct consumption by humans, for prolonged storage, used in food- or other industrial processing, and the like. The advantages of the plants, or parts thereof, according to the invention are the decreased need for fungicide treatment, thus low ring costs of material, labour, and environmental pollution, or prolonged shelf-life of products (e.g. fruit, seed, and the like) of such plants.

Any plant species or variety that is subject to some form of fungal attack may be transformed with one or more genetic constructs according to the invention in order to decrease the rate of infectivity and/or the effects of such attack. As a matter of illustration the species of the following, non-limitative, list are of

particular interest: edible flowers, such as cauliflower (Brassica oleracea), artichoke (Cynara scolymus) (edible flowers); decorative flowers, such as Chrysanthemum, lily, Rosa; edible fruit, such as apple (e.g. Malus domesticus), banana, berries (e.g. currant, Ribes rubrum), sweet ch rry (Prunus avium), cucumber (Cucumis sativus), grape (Vitis vinifera), lemon (Citrus limon), melon (Cucumis sativus), nuts (e.g. walnut Juglans regia), orange, peaches (Prunus persica), pear (Pyra communis), pepper (Solanum capsicum), prunes (Prunus domestica), strawberry (Fragaria), tobacco (Nicotiana), tomato (e.g. Lycopersicon esculentum); leaf(y) vegetables, such as cabbages (Brassica), endive (Cichoreum endivia), lettuce (Lactuca sativa), spinach (Spinacia oleraceae), leek (Allium porrum); edible roots, such as beet (Beta vulgaris), carrot (Daucus carota), turnip/swede (Brassica rapa), radish (Raphanus sativus) (edible roots); edible seeds, such as bean (Phaseolus), pea (Pisum sativum), soybean (Glycin max), wheat (Triticum aestivum), barley (Hordeum vulgare), corn (Zea mays), rice (Oryza); edible tubers, such as kohlrabi, potato (Solanum tuberosum), and the like.

The following enabling Examples serve to further illustrate the invention, and are not intended to define limitations or to restrict the scope of the subject invention.

EXPERIMENTAL

EXAMPLE 1

Assay for antifungal activity

The effect of various protein solutions on fungal growth was assessed in a microtiter plate assay. In each well of a 24-well microtiter dish 250 µl potato dextrose agar (PDA) was pipetted. Fungal spores were suspended in water and 300-500 spores in 50 µl were added to the wells. Spores were pregerminated overnight. Subsequently 100 µl filter sterilized (0.22 µm filter) protein solutions were added. As controls proteins were boiled for 10 minutes. Microtiter dishes were covered with Parafilm and incubated at room temperature. After 1-2 days the mycelium of the growing fungus in the wells was stained with lactophenol cotton blue and the extent of growth was estimated.

EXAMPLE 2

Assay for chitinase activity

Chitinase activity was assayed radiometrically with tritiated chitin as substrate (Molano et al., 1977) Tritiated chitin was synthesized by acetylation of chitosan with tritiated anhydride (Molano et al., 1977). The specific activity of the final product was approximately 1.2 x 10^5 cpm/mg. Before use the tritiated chitin was washed three times. To 100 µl 10 mM potassium phosphate buffer pH 6.4 with 0.02 % sodium azide, 50 µl tritiated chitin (approximately 150,000 cpm) and 50 µl protein solution was added. The mixture was incubated while shaking for 30 minutes at 37° C. The reaction was stopped by adding 600 µl 10% trichloro acetic acid. After centrifugation to pellet the chitin (10 minutes in a microfuge), 500 µl supernatant was filtered over glasswool and pipetted into a scintillation vial. 5 ml scintillation fluid was added and the radioactivity was counted. The amount of radioactivity released (expressed as counts per minute) was taken as a measure for chitinase activity.

EXAMPLE 3

Antifungal activity of chitinase

Antifungal activity of chitinases was assessed by the microtiter plate assay described above using the fungus Fusarium solani. Two purified extracellular tabacco chitinases (also known as pathogenesis-related proteins P and Q), a purified intracellular tobacco chitinase (32 kd protein) and a purified extracellular petunia chitinase were tested. In all cases the added activity was approximately 2000 counts per minute (meaning that this activitiy releases 2000 cpm from tritiated chitin in the chitinase assay). This activity is within in the range in which there is a linearity between protein concentration and activity. As controls bovine serum albumin (BSA), buffer or heat-inactivated chitinase was added. The r sults are shown in Table 1.

Table 1

Inhibition of the growth of Fusarium solani by chitinases.

Protein added	Inhibition
petunia extracellular chitinase	-
petunia extracellular chitinase, boiled	-
tobacco extracellular chitinase (PR-P)	-
tobacco PR-P, boiled	-
tobacco extracellular chitinase (PR-Q)	-
tobacco PR-Q, boiled	-
tobacco intracellular 32 kd chitinase	+
tobacco 32 kd intracellular chitinase, boiled	-
BSA	-
buffer	-

- : no inhibition; + : inhibition

From the results in Table I it can be concluded that the extracellular chitinases of tobacco and of petunia do not possess antifungal activity.

EXAMPLE 4

4.0 The cloning of cDNAs corresponding with chitinase

Polyadenylated RNA was isolated from TMV-infected Samsun NN tobacco and double stranded cDNA was made using oligo(dT) as a primer (Hooft van Huijsduijnen et al., 1986) using standard techniques known to researchers in this area. The double stranded DNA was provided with "C-tails" which were hybridized with "G-tails" which were brought into the plasmid pUC9 after this plasmid was spliced open with PstI (Maniatis et al., 1982). The constructs obtained were used for the transformation of Escherichia coli MH-1. The transformants were brought in duplo on nitrocellulose filters. The first filter was hybridized in vitro with transcribed cDNA of poly(A)-RNA from TMV-infected tobacco, the other filter was hybridized with cDNA against poly(A)-RNA from healthy tobacco (Maniatis et al., 1982). Transformants which showed better hybridization with the first probe than with the second contained cDNA corresponding with mRNAs whose synthesis was induced via the TMV infection. The cDNA clones obtained could be subdivided into six clusters on the basis of cross-hybridizations of the insertions: within a cluster, the insertions of all clones hybridize with each other, between clusters no cross-hybridizations took place (Hooft van Huijsduijnen et al., 1986) under the hybridization and wash conditions used (0.1 SSC, 1% SDS, 65°C; Maniatis et al., 1982). The TMV-inducibility of the synthesis of mRNAs corresponding with the insertions of the clones of the six clusters, were confirmed via Northern blot analyses, well known to researchers in this area (Hooft van Huijsduijnen et al., 1986).

Via immunoprecipitations of in vitro translation-products of mRNAs by means of selective hybridization with (the insertions of) cDNA clones from the six clusters, it was established that the clones of two clusters, namely clusters D and F, correspond with mRNAs for proteins serologically related to the so-called PR-proteins P and Q (Hooft van Huisduijnen et al., 1987). The experiments were conducted according to standard techniques known to researchers in this area. The PR-proteins P and Q were already earlier identified as extracellular acidic chitinases, and antibodies against both proteins cross-react with two basic chitinases also present in tobacco (Legrand et al., 1987). Inserts of clones from clusters D and F were subcloned in M13-vectors and the sequence of the insertions was determined by the method of Sanger et al. (1977). One clone from cluster F, namely PROB3, appeared to contain an insertion of 412 base-pairs, wherein an open reading fram occurs, coding for 109 amino acids wherefrom the sequence appears to be identical to the C-terminal sequence of a basic chitinase of tobacco (Hooft van Huijsduijn n t al., 1987). The amino acid sequ nce of this chitinase was determined from th nucleotide-sequence of a cDNA clone, namely pCHN50 (Shinshi et al., 1987). Cluster F, including clon PROB3, consequently corresponds with one or more intracellular basic chitinases of tobacco.

Clust r D contains on clone, namely PROB30, with an insertion of 404 base-pairs, wherein an open reading fram occurs, coding for 67 amino acids (Hooft van Huijsduijnen et al., 1987). The homology betw en th amino acid sequ nces deduced from the nucleotide-sequ nces of the ins rtions of PROB3 and PROB30 appears to be 65%, while the nucleotide-sequences themselves showed a homology of only 56%. From this it was concluded that PROB30 corresponds with a chitinase that is related to, but is not identical to the intracellular chitinase. After partial amino acid sequences for PR-proteins P and Q were established, it was concluded that PROB30 corresponds with PR-protein P, an extracellular acidic chitinase of tobacco.

4.1 Construction of cDNA clones coding for an entire extracellular chitinase

To obtain cDNA clones containing the entire coding sequence for the chitinases, clone PROB30 was used as a probe for the selection of clones from a Petunia hybrida cDNA library. Double stranded cDNA was synthesized as described above, treated with EcoRI-methylase, provided with EcoRI-linkers, ligated to lambda gt11 vector-arms and transfected to E. coli Y1090 entirely according to the method described in the instruction manual belonging to "cDNA cloning system-lambda gt11" (Amersham International plc, 1986). Afterwards, the newly constructed library was searched with the plaque hybridization-technique of Benton and Davis (1977) whereby the previously described acidic chitinase-cDNA clone served as a probe. In this manner, five recombinant phages were obtained with sequences homologous to PROB30. Recombinant phage DNA was isolated and afterwards the insertions were spliced out with EcoRI and subcloned in a pUC plasmid, resulting in the clones, D1, D2, D5, D6 and D8. After being subcloned in sections into M13-phages, the nucleotide sequences of the original insertions were entirely or partially determined. In Figure 1, the sequence of clone D1 and a deduced amino acid sequence are provided. The first and the last 7 nucleotides originate from the EcoRI-linkers which were used for the construction of the library. The sequence of eight A-residues at the end of the insertion, just before the EcoRI recognition site represent the remainder of the poly(A)-tail of the original mRNA and consequently confirms the orientation of the insertion previously assigned through the large open reading frame and the homology to the deduced amino acid sequence of other chitinases (see above). The insertion of clone D5 appears to be 10 nucleotides longer on its 5' extremity than that of D1; the remainder of the poly(A)-tail was however, as with the insertion of D6, found 25 nucleotides earlier in the sequence. For as far as could be traced, the sequences of the insertions of D8, D2 and D6 appeared to be identical to those of D1 and D5.

The homology between the determined amino acid sequence of Petunia clone D1 and tobacco clone PROB30 is approximately 80%. PROB30 is a partial cDNA clone which corresponds with PR-protein P, an extracellular chitinase. Analyses of transgenic plants have proven that the chitinase encoded on D1 is extracellularly localized, at least in tobacco. D1 consequently contains the entire nucleotide sequence coding for an extracellular chitinase.

In order to clone the cDNA corresponding with the extracellular chitinase on a BamHI fragment, the following experiments were performed.

Two of the oligonucleotides were synthesized, namely 5'-AGCTTGGATCCGTCGACGGTCCT-3' and 5'-AATTAGGATCCGTCGACGGATCCA-3', and these were hybridized to one another, resulting in a double stranded DNA fragment with one extremity compatible with the HindIII recognition site and one extremity compatible with the EcoRI recognition site. Furthermore, the fragment contains recognition sites for BamHI, HincII and once again, BamHI. This fragment is cloned in pUC19, spliced open with EcoRI and HindIII, whereby the HindIII recognition site is restored but the EcoRI recognition site is not.
The new plasmid was called pUC19+. After the extremities of the EcoRI insertion of clone D1 were filled in with Klenow polymerase according to standard techniques, the fragment was cloned into the HincII site of pUC19+.

4.2 Construction of a cDNA clone coding for an entire intracellular chitinase

Screening of a new Samsun NN library (which was constructed in the same manner as the Petunia library described above) with the PROB3 insertion provided a recombinant phage. The insertion of this phage was subcloned into a plasmid as a EcoRI fragment, resulting in clone F1. Clarification of the primary structure showed that the nucleotide sequence of the insertion of F1 was identical to clone pCHN50, which was characterized by Shinshi and co-workers (1987). Because the insertion of pCHN50 has been characterized as a s quence corresponding with the intrac llular chitinase of tobacco, it was concluded that the insertion of F1 also corresponds to an intracellular chitinase. Th ins rtion of pCHN50 does not contain the entire coding sequence and is consequently incomplete. Although the insertion of F1 is 30 nucleotides longer on the 5' extremity than is pCHN50, the chitinase coding sequence contained in F1 is also

incomplete.

To obtain a fragment with a sequence which codes for an entire chitinase, the following cloning steps were performed. The insertion f F1 was cloned as an EcoRI fragm nt into pIC19H (Marsh et al., 1984) such that the 3' extremity of the insertion properly came to the BamHI site of the polylinker. This resulted in plasmid pIC19/F1.

Two oligonucleotides were synthesized (5'-GATCCAACATGAGGCTGTGCA-3' and 5'-AATTTGCACAGCCTCATGTTG-3') which form a fragment after hybridization to each other. This fragment is cloned in a three-point ligation reaction in a pUC plasmid spliced open with BamHI-PstI, together with the EcoRI-PstI fragment, with the 5' extremity of the open reading frame in the insertion of pIC19/F1. This cloning results in pUC/5'F1. The sequence of the oligonucleotides was chosen such that the fragment coded for five amino acids, and also such that in the eventually obtained BamHI-PstI fragment, the EcoRI recognition site was eliminated and the triplets for said five amino acids were in phase with the open reading frame in F1. After digestion of pIC19H/F1 with HindIII and (partially) with PstI, the HindIII/PstI fragment was cloned with the 3' part of the insertion into an intermediate vector lacking a EcoRI recognition site. The EcoRI site on the extremity of the insertion was replaced by filling in and back-ligation, techniques known to researchers in this area. After elimination of the EcoRI recognition site, the HindIII-PstI fragment was cloned into pUC/5'F1. The thus-obtained plasmid contains on a BamHI fragment, a cDNA with an entire coding sequence for an intracellular chitinase from tobacco. In Figure 2, the sequence of this BamHI fragment with the deduced amino acid sequence are provided. The sequence of nucleotides 2 through 22 originates from the synthetic fragment, while nucleotides 23-27 form the remainder of the EcoRI recognition site. The PstI recognition site (5'-CTGCAG-3') is found at position 129-134. The last 21 nucleotides of this sequence successively represent a filled in EcoRI recognition site which originates from an EcoRI linker-molecule used for the construction of the cDNA library, a SmaI and a BamHI recognition site, both originating from the polylinker of pIC19H.

4.3 Construction of a gene encoding an intracellular chitinase, modified as to obtain apoplast targeting of the protein.

For the construction of a gene coding for an intracellular chitinase to be targeted to the apoplast, the sequence of the intracellular chitinase gene as shown in Figure 2 was modified. The G at position 961 was changed into a T, hence creating a stopcodon. A second stopcodon was introduced by the replacement of the T residue at position 968 into an A. The change of the T residue at position 975 into a C resulted in the creation of a SalI-site. These modifications were introduced by using an overlapping polymerase chain reaction (PCR) technique, known to persons skilled in the art. Afterwards, the whole sequence was checked for possible introduction of mutations as a result of the PCR technique.

EXAMPLE 5

5.0 Cloning of genes coding for extra- and intracellular glucanases

The previously described lambda gt11 tobacco cDNA library was screened for recombinant phages expressing PR-2, PR-N or related sequences, with antiserum, obtained from rabbits that were immunized with tobacco PR-proteins 2 and N. The technique used was based on methods described by Huynh et al., (1985) and may be presumed to be known by researchers in this area. The insertion of one recombinant phage identified by this method, was used as a probe to rescreen the library, but this time using the plaque hybridization technique of Benton & Davis (1977). Using this method, 30 recombinant phages were identified. The insertions in the DNA of these phages were spliced open with EcoRI and subcloned into a pUC plasmid. On the basis of their various restriction patterns, the thus-obtained clones were divided into a number of groups. After subcloning in M13 vectors, the nucleotide sequences of a number of clones from each group were determined, and the amino acid sequences of the peptides encoded thereby was deduced. These analyses, in combination with the comparison of the thus-obtained sequences to sequences previously known, indicate that for at least 5 groups of clones, each codes for a unique β-1,3-glucanase. Hybridization experiments with total RNA from tobacco, whereby one of the glucanase cDNAs was used as a probe, showed that th se glucanase mRNAs were also synthesized following induction with salicylate or following TMV-infection (Memelink t al., 1989).

5.1 Isolation of genes coding for extracellular β-1,3-glucanases

Using on of th above described cDNA clon s, a genomic library of DNA from th nucleus of Samsun NN tobacco partially spliced with Sau3AI (Cornelissen et al., 1987), screened on recombinant phages with genes coding for glucanases. A number of recombinant phages were btained from which four, namely gl1, gl3, gl4 and gl9 (PR-N), were furth r charact rized. South rn blot analys s resulted in restriction maps which showed that each of the four clones contained an unique gene. After subcloning in successive pUC plasmids and M13 vectors, sequence analyses were carried out on gl3 and gl9. The sequence of the gene on clone gl9, together with the amino acid sequence deduced therefrom, are provided in Figure 3. Comparisons teach that this amino acid sequence is identical to that of the tobacco extracellular β-1,3-glucanase PR36, the amino acid sequence of which was partially clarified (Van den Bulcke et al., 1989) with the understanding that the 21st amino acid on the C-terminal end, a threonine residue, appeared not to be present in PR36. The gene herein described is the first isolated and characterized DNA sequence coding for an extracellular β-1,3-glucanase.

To clone the gene in an expression vector, the following treatments were carried out. Using the PCR technique, known to researchers in this area, a BamHI recognition site was introduced before the gene and a HindIII recognition site was introduced after the gene. Afterwards the sequence is checked for possible introduction of mutations as a result of the PCR technique. After ligation of the gene as a BamHI-HindIII fragment into expression vector pMOG183 (see under 6), and following linearization by splicing with BamHI and HindIII, an expression unit arises on a SstI-HindIII fragment with the transcription terminator of the glucanase gene itself.

5.2 Isolation of genes coding for intracellular glucanases

A clone corresponding to an intracellular glucanase (Memelink et al., 1989) is used as a probe to search the above described genomic library. Though a large number of recombinant phages with unique insertions were obtained, it appeared after restriction analysis that only 2 unique genes are concerned. The DNA from one phage, gGLB50, was further characterized by Southern blot analysis, subcloning and via clarification of the primary structure of insertions of the relevant subclones, all of which was done using techniques known to researchers in this area. The primary structure of the gene as eventually obtained, together with the amino acid sequence deduced therefrom, are provided in Figure 4. Comparisons teach that this amino acid sequence is extremely homologous to the sequence of an intracellular, basic β-1,3-glucanasefrom tobacco such as deduced from the sequences of a number of overlapping cDNA clones by Shinshi and co-workers (1988). Though the cDNAs possibly correspond to one another with strong relation, they are nevertheless different genes. At least one of the cDNA clones contains an insertion having a sequence identical to a part of the herein described gene.

To clone the gene into an expression vector, the following steps were carried out. Using the PCR technique, known by researchers in this area, a BamHI recognition site is introduced before the gene and an SstI recognition site is introduced after the gene. Afterwards, the sequence is checked for possible introduction of mutations as a result of the PCR technique. Following ligation of the gene as a BamHI-SstI fragment in expression vector pMOG185 (see under Example 6), after linearization of the vector by digestion with BamHI and SstI, an expression unit arises on a XbaI-SstI fragment with the transcription terminator of the glucanase gene itself.

In addition to the above, using the PCR technique, a BamHI recognition site is introduced before the gene. Afterwards, the sequence is checked for possible introduction of mutations as a result of the PCR technique. Subsequently, the BamHI-XbaI fragment conataining the glucanase gene was cloned into plasmid pIC19H (Marsh et al., 1984), after linearisation of the plasmid by digestion with BamHI and XbaI. After linearization of expression vector pMOG183 (see under 6) by digestion with BamHI and HindIII, the gene was ligated into this vector as a BamHI-HindIII fragment, resulting in a glucanase expression unit on a SstI-HindIII fragment with the transcription terminator of the glucanase gene itself.

5.3 Construction of a gene encoding an intracellular glucanase, modified as to obtain apoplast targeting of the protein.

For the construction of a gene coding for an intracellular glucanase to be targeted to the apoplast, modifications were made in the sequence of the intracellular glucanase gene described under 5.2. To this end the sequence GTCTCTGGTGG (nucleotides 1883-1893 in Figure 4) was changed into the sequence TGATATCGTTA using the PCR technique. This modification results in the introduction of two stopcodons with an EcoRV recognition site inbetween. Sequences were checked for possible introduction of mutations as a result of the PCR technique.

EXAMPLE 6

6.0 Construction of expression vectors

A high constitutive expression of genes is pending upon, inter alia, the promoter of the genes concerned. To satisfy such demands, expression vector pMOG181 was constructed, and is depicted in Figure 5. To this end, the expression cassette of pROK1 (Baulcombe et al., 1986) is cloned in pUC18 as a EcoRI-HindIII fragment. This cassette contains the cauliflower mosaic virus (CaMV) 35S promoter on an EcoRI-BamHI restriction fragment and the nopaline synthase (nos) transcription terminator on a BamHI-HindIII fragment. The promoter fragment consists of the sequence beginning with the -800 residue and extending to and including the +1 residue of the CaMV genome, whereby position +1 is the transcription initiation site (Guilley et al., 1982). From the literature it is known that the duplication of the sequence between -343 and -90 increases the activity of the CaMV 35S promoter (Kay et al., 1987). To obtain a promoter fragment with a double so-called enhancer sequence, the following cloning steps were carried out using techniques known to researchers in this area. First, the sequence upstream from the NcoI recognition site at position -512 was deleted and the NcoI recognition site itself was changed into an EcoRI recognition site. To achieve this, the expression cassette in pUC18 was spliced open with NcoI, the thus-obtained extremities were filled in with Klenow polymerase and an EcoRI linker was ligated into the extremities. The plasmid obtained was spliced open with EcoRI, resulting in the deletion of the EcoRI fragment, and the extremities were filled in using Klenow polymerase. Afterwards, the filled in AccI-EcoRV promoter fragment (position -388 to -90) was cloned into the linear plasmid, whereby the ligation of the filled EcoRI to the filled-in AccI recognition site created a new EcoRI site. The newly obtained plasmid, pMOG181, contains the CaMV 35S promoter with double enhancer sequences in an expression cassette which still lies on an EcoRI-HindIII fragment.

A number of derivatives were made from pMOG181. An adaptor (5'-AATTGAGCTC-3') was cloned into the EcoRI recognition site, such that the EcoRI site was not recovered and a SstI recognition site was introduced. The resulting plasmid, pMOG183 (Figure 6), now contains the expression cassette of a SstI-HindIII fragment. In the same manner, pMOG184 was developed from pMOG181 (Figure 7) by the replacement of the HindIII site with a SstI recognition site. Replacement of the EcoRI site in pMOG184 by a XbaI site provided pMOG185 (Figure 8).

EXAMPLE 7

Binary vectors

In order to introduce the chimeric chitinase and β-1,3-glucanase genes into the genome of tobacco via Agrobacterium tumefaciens, the binary vectors pMOG23 (Figure 9) and pMOG22 (Figure 10) were used. Vector pMOG23 is a derivative of vector BIN19 (Bevan, 1984). In view of this last vector, the following changes were made, which are not essential for the invention, using techniques known to researchers in this area. In the first place, the positions of the left border (LB) and the right border (RB), in view of the neomycine phosphotransferase gene II (NPTII gene), are exchanged for each other. Afterwards, the orientation of the NPTII gene is turned around such that the transcription of the gene occurs in the direction of the LB. Finally the BIN19 polylinker is replaced with a polylinker with the following restriction enzyme recognition sites: EcoRI, KpnI, SmaI, BamHI, XbaI, SacI, XhoI and HindIII.

Vector pMOG22 is a derivative of pMOG23 wherein the NPTII gene is replaced with a hygromycine resistance gene. The gene used codes for a Escherichia coli hygromycine phosphotransferase (HPT) and is taken from plasmid PLG90 (Van den Elzen et al., 1985). This plasmid is a derivative of pLG88 (Gritz et al., 1983) and contains a BamHI recognition site extending from 19 base pairs before the translation initiation codon to 20 base pairs after the stop codon of the gene. Using site directed mutagenesis, a standard recombinant DNA technique known to researchers in this area, the ATG codon four nucleotides before the translation initiation codon is changed into an ATA codon. In the same manner, the EcoRI recognition site in the coding region of the HPT gene is changed to 5'CAATTC 3'. Afterwards, the BamHI fragment, following filling in of both BamHI extremities using Klenow polymerase (Maniatis et al., 1982), is cloned in the BamHI recognition site of the expression cassette of pROKI (Baulcombe et al., 1986) after both BamHI extremities wer also filled in. In this mann r an expression unit was obtained with the HPT coding sequence between the CaMV 35S promoter and the nos transcription terminator.

EXAMPLE 8

Cloning chimeric genes in binary vectors

The cDNA coding for the extracellular chitinas (described in 4.1), the intracellular chitinase cDNA (described in 4.2) and the modified intracellular chitinase cDNA (described in 4.3) are cloned as BamHI fragments in pMOG181. Clones are selected, using restriction enzyme analysis, which had the coding sequences in the proper orientation after the promoter. Afterwards the expression cassettes isolated as EcoRI-HindIII fragments were cloned into the binary vector pMOG23, following linearisation of this plasmid this plasmid with EcoRI and HindIII. The resulting plasmids are called pMOG196, pMOG198 and pMOG189, respectively. In addition, the expression cassette with the gene encoding the intracellular chitinase modified as to target the protein to the apoplast, is cloned into pMOG22 as well, resulting in pMOG289.

The SstI-HindIII fragment with the expression unit for the intracellular glucanase modified as to target the protein to the apoplast (described in 5.3), is cloned into pMOG23, resulting in pMOG512.

The cDNA coding for the extracellular chitinase is cloned as a BamHI fragment in pMOG184 and the cDNA coding for the intracellular chitinase as a BamHI fragment in pMOG183. Following both cloning steps, clones are selected using restriction enzyme analysis which placed the coding sequences in the proper orientation after the promoter. Afterwards, both cassettes were isolated as EcoRI-SstI and SstI-HindIII fragments, respectively, and in a three point ligation, cloned in pMOG23, following linearization of this plasmid with EcoRI and HindIII. The plasmid obtained, pMOG200 (Figure 11), now contains both chitinase genes on a binary plasmid physically coupled to the NPTII gene.

The SstI-HindIII fragment with the expression unit for the extracellular glucanase and the XbaI-SstI fragment with the expression unit before the intracellular glucanase are cloned in a three point ligation reaction into the binary vector pMOG22, following linearization of this plasmid with XbaI and HindIII. The obtained binary plasmid, pMOG212 (Figure 12) now contains both glucanase genes physically coupled to the hygromycine resistance gene.

EXAMPLE 9

Transgenic plants

For the transformation of tobacco, use is made of leaf-discs (Horsch et al., 1985) originating from axenically cultured plants. The cultivation was performed with bacterium strains, derived from Agrobacterium tumefaciens strain LBA4404 (CBS 191.83; Hoekema et al., 1983) wherein a binary vector was crossed in by means of mobilisation with the help from the plasmid pRK2013 (Ditta et al., 1980). The thus-obtained Agrobacterium strains were maintained under selection pressure (20 mg/L rifampicine, 100 mg/l kanamycin), and was cultured as such for co-cultivation. The formation of transgenic shoots was established on media with 100 mg/l kanamycin in cases where derivatives of the binary vector pMOG23 were used, and on media with 20 mg/l hygromycin if derivatives of pMOG22 were used. The transgenic plants obtained from the shoots were analyzed for the expression of the newely introduced genes , using the so-called Western blotting technique. The Western blotting technique is known to researchers in this area. In some cases leaf-discs were taken from transgenic plants to insert additional genes. Kanamycin resistant leaf-discs were cocultivated with Agrobacterium strains containing pMOG22 derivatives, and hygromycin resistant leaf-discs were co-cultivated pMOG23 derivatives. The plants capable of the constitutive expression of all the introduced genes were selected, and seeds were obtained after they were fertilized via self-pollination. F1-seedlings of these transgenic plants were used for further analysis.

Transgenic tobacco plants were obtained transformed with either pMOG196, pMOG198, pMOG189 or pMOG512, and double transformed with either pMOG196 + pMOG289, pMOG512 + pMOG289 or pMOG200 + pMOG212.

EXAMPLE 10

Targeting intracellular chitinase to the apoplast

To evaluate the possibility to target the intracellular chitinase to the apoplastic space, the following experiment was carried out.

Samsun NN tobacco plants were tranformed with pMOG196 to constitutiv ly express the Petunia extracellular chitinase gene (plant line 1); with pMOG198 to constitutively express the tobacco intracellular chitinase gene (line 2); with pMOG189 to constitutively express the modified intracellular chitinase gene (line 3) and with pMOG196 + pMOG289 to constitutively express the extracellular chitinase gene and the

intracellular chitinase gene modified to obtain targeting to the apoplastic space. The lines of transgenic plants were selected for high expression of each chimeric gene (up to 0.5% of total soluble protein fraction was reached). From each of the four selected lin s both extracellular fluid (isolati n procedure, vide: Parent & Asselin, 1984) and total leaf protein-extracts were prepared (Kaufmann t al., 1987) and these were tested for antifungal activity on the fungus Fusarium solani. Chitinsase activity was detected in the extracellular fluid (EF) of plant lines 1, 3, and 4, and in the total protein extract (TE) of all plant lines (1 to 4). In the antifungal assay 100 µl of EF from lines 1, 3 and 4 were added, diluted to contain a chitinase activity of approximately 2000 cpm (see example 2). The dilutions of EF of line 2, and the non-transgenic control tobacco were the same as the dilution of line 1. The 100 µl of the diluted TE of the four transgenic lines contained a chitinase activity of approximately 2000 cpm. The dilution of TE of the control was equal to that of plant line 1. The results of the antifungal assay are given in Table 2.

Table 2

Inhibition of growth of *Fusarium solani* by chitinases from transgenic tobacco plants

Transgenic plant	Inhibition	
	Extracell. fluid	Total extract
line 1 (extracell.)	-	-
line 2 (intracell.)	-	+
line 3 (mod.intracell.)	+	+
line 4 (extracell.+ mod. intracellular)	+	+
non-transformed	-	-

- : no inhibition; + : inhibition

Neither the EF nor the TE of line 1, expressing the Petunia extracellular chitinase gene, shows any antifungal effect, as expected from the experiments using the purified chitinases (see Example 3). The presence of chitinase activity in the EF of line 1 shows that the Petunia chitinase in tobacco is targeted to the apoplastic space.

Although in most experiments neither chitinase activity nor antifungal activity could be detected in the EF of line 2, in some experiments chitinase activity was found in the EF, probably due to leakage of the (relatively over-) expressed intracellular chitinase from the cells. The TE of line 2 showed a strong antifungal effect. Of line 3, expressing the modified apoplast-targeted intracellular chitinase gene, both the EF and the TE exhibited a strong antifungal effect. This proves that the targeted intracellular chitinase of line 3 still has antifungal activity. Apparently, deletion of (part of) the C-terminal vacuole-targeting signal does not significantly affect the antifungal activity of the intracellular chitinase.

EXAMPLE 11

synergistic effect of glucanase on antifungal activity of chitinase

Samsun NN tobacco plants were transformed with pMOG512 to constitutively express the modified intracellular glucanase gene(line 1); with pMOG512 + pMOG289 to constitutively express the modified intracellular chitinase gene and the modified intracellular glucanase gene (line 2) and with pMOG189 to express the modified intracellular chitinase gene (line 3; see example 10). The plant lines were selected for high levels of expression of each chimeric gene. From each of the selected lines extracellular fluid (EF) (Parent & Asselin, 1984) and total leaf protein extracts (TE) (Kaufmann et al., 1987) were prepared. Initial dilutions were mad of EF and TE of lines 2 and 3 to contain a chitinase activity of approximately 2000 cpm (see example 2). The initial dilutions of EF and TE of line 1 were equal to those of line 3. Subsequently, dilution series were made of the initial dilutions and these wer tested for antifungal activity. No difference was found in antifungal activity between diluti n series of EF and of TE. Moreover the highest antifungal

activity was found in the (diluted) extracts of line 2. Apparently, the apoplast-targeted intracellular glucanase has a synergistic effect on the antifungal activity of the apoplast-targeted intracellular chitinase.

EXAMPLE 12

Analysis of transgenic plants having combined expression of an unmodified intracellular chitinase and glucanase and an extracellular chitinase and glucanase

Phytophthora nicotianae var. nicotianae (Pnn) is a fungus which belongs to the family of Oomycetes. It is a root pathogen of tobacco, inter alia. The infection of this plant leads to the wilting of leaves and/or to rotting in the base of the stem (black shank). Eventually the tobacco plant perishes from the infection.

To evaluate the fungal resistance of transgenic plants, that express unmodified genes encoding plant hydrolytic enzymes, the following experiment can be performed. Ten transgenic plants constitutively expressing the two unmodified chitinase and the two unmodified β-1,3-glucanase genes (the unmodified chitinase gene from pMOG 200; the unmodified β-1,3-glucanase gene from pMOG212), ten control plants transformed with the empty vector and ten non-transgenic plants are inoculated with a suspension in water of $2x10^5$ Pnn zoospores. The suspension is pipetted onto the base of the stem in the soil in the pot wherein the plant is grown and thereafter rinsed with water. In the time thereafter, the plants are monitored for the development of disease symptoms. After two to three days, the control plants and the non-transgenic plants will show the first disease symptoms; after 3 weeks, approximately 17 of the 20 plants will show symptoms; a few plants will be dead. Of the transgenic plants that constitutively express the two chitinase and β-1,3-glucanase genes, just a few plants will show a slight wilting after 3 weeks.

In an alternative experiment, leaf-discs having a diameter of approximately 1 cm can obtained from the leaves of transgenic plants capable of the constitutive expression of both chitinases and both glucanases, from control transgenic plants and from non-transgenic tobacco plants. Subsequently, 10 μl of a Pnn zoospore suspension in water (5000 zoospores per ml) is pipetted onto the (underside of the) disks, and afterwards the disks are placed in sterile water and allowed to incubate at room temperature. Three sets of five disks can be used in each test, thus in total, ten control disks per experiment. The experiment can be carried a number of times with the same consistent result. After about a day, the first signs of a beginning infection will be observed on the control disks. After five days, they will be fully colonized. The disks of the transgenic plants capable of expressing chitinases and glucanases will show less severe disease symptoms, even after five days.

Tests with leaf disks, performed precisely as described above, can also performed with spores of the fungus Thanatephorus cucumeris (anamorph Rhizoctonia solani Kuhn), a basidiomycete. The inoculum concentration used can be 5000-10000 basidiospores per ml water. After ten days the disks are checked. The disks from the non-transgenic plants and the control transgenic plants will all appear to be infected, while the disks from the transgenic plants, expressing the chimeric chitinase and β-1,3-glucanase genes will be much less affected.

Likewise, the sensitivity of transgenic and control plants can be tested on the fungus Alternaria alternata, an Ascomycete. This fungus causes "brown-spot" in tobacco. The experiments can be performed in the manner as described by Spurr in 1973. The inoculum concentration used can be 5000-10000 conidia per ml. After 10 days, the development of "brown-spot" on the inoculated leaf material is judged according to the criteria suggested by Spurr (1973). The non-transgenic and the control leaf-material will show light to very heavy necroses, while the leaf-material having a constitutive expression of both chitinase and both β-1,3-glucanase genes will show no, or much less severe disease symptoms (light yellow lesions).

If the experiments are carried out as described above, they will show, that the constitutive expression of an extracellular chitinase and an extracellular β-1,3-glucanase and an intracellular chitinase and an intracellular β-1,3-glucanase provides a resistance against, at least a reduced susceptibility or sensitivity for fungal infections.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually incorporated by reference.

Although the foregoing invention has been described in som detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scop of the appended claims.

DEPOSIT OF MICROORGANISMS

The Escherichia coli strain DH5α, containing the plasmid pMOG23 (CBS 102.90) and the Escherichia coli strain DH5α containing th plasmid pMOG22 (CBS 101.90) were deposited on January 29, 1990, at the Centraal Bureau voor Schimmelcultures (CBS), Baarn, the Netherlands. The genotype of Escherichia coli strain DH5α is : F^-, endA1, hsdR17 ($r_k^-m\ m_k^+$), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, φ80dlacZ M15.

REFERENCES

Abeles F.B. et al. (1971), Plant Physiol. **47**, 129-134.
Ballance G.M. et al. (1976), Can. J. Plant Sci. **56**, 459-466.
Baulcombe D.C. et al. (1986), Nature **321**, 446-449.
Bauw G. et al. (1987), Proc. Natl. Acad. Sci. USA **84**, 4806-4810.
Bell J.N. et al. (1986), Mol. Cell. Biol. **6**, 1615-1523.
Benton W.D. & Davis R.W. (1977), Science **196**, 180-182.
Bevan M. (1984), Nucl. Acids Res. **22**, 8711-8721.
Boller T. et al. (1983), Planta **157**, 22-31.
Boller T. & Métraux J.P. (1988), Mol. Plant Pathol. **33**, 11-16.
Boller T. & Vögeli U. (1984), Plant Physiol. **74**, 442-444.
Broglie K.E. et al. (1986), Proc. Natl. Acad. Sci. USA **83**, 6820-6824.
Broglie K.E. et al. (1989), Plant Cell **1**, 599-607.
Castresana et al. (1988), Embo J. **7**, 1929-1936.
Chappell J. et al. (1984), Planta **161**, 475-480.
Christ U. & Mösinger E. (1989), Physiol. Mol. Plant Pathol. **35**, 53-65.
Cohen Y. & Kuc J. (1981), Phytopathology **71**, 783-787.
Cornelissen B.J.C. et al. (1987), Nucl. Acids Res. **15**, 6799-6811.
Coruzzi et al. (1984), Embo J. **3**, 1671-1679.
Cramer C.L. et al. (1985), EMBO J. **4**, 285-289.
Cruickshank I.A.M. & Mandryk M. (1960), Aust. Inst. Agric. Sci. **26**, 369-372.
Darvill A.G. & Albertsheim P. (1984), Plant Physiol. **35**, 243-275.
De Loose M. et al. (1988), Gene **70**, 13-23.
De Wit P.J.G.M. et al. (1986), Physiol. Mol. Plant Pathol. **29**, 159-172.
De Wit P.J.G.M. & Van der Meer F.E. (1986), Physiol. Mol. Plant Pathol. **28**, 203-214.
Ditta G. et al. (1980), Proc. Acad. Natl. Sci. USA **77**, 7347-7351.
Elliston J. et al. (1977), Phytopathol. Z. **87**, 289-303.
Felix G. & Meins F. (1985), Planta **164**, 423-428.
Felix G. & Meins F. (1986), Planta **167**, 206-211.
Felix G. & Meins F. (1987), Planta **172**, 386-392.
Ferraris L. et al. (1987), J. Phytopathol. **118**, 317-325.
Fincher G.B. et al. (1986), Proc. Natl. Acad. Sci. USA **83**, 2081-2085.
Fink W. et al. (1988), Plant Physiol. **88**, 270-275.
Fischer W. et al. (1989), Physiol. Mol. Plant Pathol. **35**, 67-83.
Fortin M.G. et al. (1985), Can. J. Bot. **63**, 932-937.
Fry (1986), Ann. Rev. PLant Phys. **37**, 765-786.
Gaynor J.J. (1988), Nucl. Acids Res. **16**, 5210.
Gessler G. & Kuc J. (1982), J. Exp. Bot. **33**, 58-66.
Gilpatrick J.D. & Weintraub M. (1952), Science **115**, 701-702.
Guilley H. et al. (1982), Cell **30**, 763-773.
Gritz L. & Davies J. (1983), Gene **25**, 179-188.
Hadwiger L.A. & Beckman J.A. (1980), Plant Physiol. **66**, 205-211.
Hecht E.I. & Bateman D.F. (1964), Phytopathology **54**, 523-530.
Hedrick S.A. et al. (1988), Plant Physiol. **86**, 182-186.
Hilborn M.T. & Farr W.K. (1959), Phytopath. **70**, 35-39.
Hoekema A. et al. (1983), Nature **303**, 179-180.
Hoj P.B. et al. (1989), Plant Mol. Biol. **13**, 31-42.
Hoj P.B. et al. (1981), J. Inst. Brew. **87**, 77-80.
Hoj P.B. et al. (1988), FEBS Lett. **230**, 67-71.
Hooft van Huijsduijnen R.A.M. et al. (1986), EMBO J. **5**, 2057-2061.
Hooft van Huijsduijnen R.A.M. et al. (1987), Plant Mol. Biol. **9**, 411-420.
Horsch R.B. et al. (1985), Science **227**, 1229-1231.

Hunsley D. & Burnett J.H. (1970), J. Gen. Microbiol. **62**, 203-218.
Jamet E. & Fritig B. (1986), Plant Mol. Biol. **6**, 69-80.
Jones D. et al. (1974), Biochem. J. **140**, 47-55.
Joosten M.H.A. & De Wit P.J.G.M. (1989), Plant Physiol. **89**, 945-951.
Kauffmann S. et al. (1987), EMBO J. **6**, 3209-3212.
Kay R. et al. (1987), Science **236**, 1299-1302.
Keen N.T. & Yoshikawa M. (1983), Plant Physiol. **71**, 460-465.
Kombrink E. & Hahlbrock K. (1986), Plant Physiol. **81**, 216-221.
Kombrink E. et al. (1988), Proc. Natl. Acad. Sci. USA **85**, 782-786.
Kuc J. (1982), Wood R.K.S. ed. pp. 157-178. Plenum Press, New York.
Leah R. et al. (1987), Carlsberg Res. Commun. **52**, 31-37.
Laflamme D. & Roxby R. (1989), Plant Mol. Biol. **13**, 249-250.
Legrand M. et al. (1987), Proc. Natl. Acad. Sci. USA **84**, 6750-6754.
Lotan T. et al. (1989), Plant Cell **1**, 881-887.
Mandryk M. (1963), Aust. J. Agric. Res. **14**, 315-318.
Maniatis T. et al. (1982), Cold Spring Harbor Laboratory, CSH, New York.
Marsh J.L. et al. (1984), Gene **32**, 481-485.
Mauch F. et al. (1984), Plant Physiol. **76**, 607-611.
Mauch F. et al. (1988a), Plant Physiol. **87**, 325-333.
Mauch F. et al. (1988b), Plant Physiol. **88**, 936-942.
Mauch F. & Staehelin L.A. (1989), Plant-Cell **1**, 447-457.
Mazau D. & Esquerré-Tugayé M.T. (1986), Physiol. Mol. Plant Pathol. **29**, 147-157.
McIntyre J.R. & Dodds J.A. (1979), Physiol. Plant Pathol. **15**, 321-330.
McIntyre J.L. et al. (1981), Phytopathology **71**, 297-301.
Meins F. & Ahl P. (1989), Plant Science **61**, 155-161.
Memelink J. et al. (1987), EMBO J. **6**, 3579-3583.
Memelink J. et al. (1990), Plant Mol. Biol. **14**, 119-126.
Métraux J.P. & Boller T. (1986), Physiol. Mol. Plant Pathol. **28**, 161-169.
Métraux J.P. et al. (1988), Physiol. Mol. Plant Pathol. **33**, 1-9.
Métraux J.P. et al. (1989), Proc. Natl. Acad. Sci. USA **86**, 896-900.
Mohnen D. et al. (1985), EMBO J. **4**, 1631-1635.
Molano J. et al. (1977), Chitin. Anal. Biochem. **83**, 648-656.
Moore A.E. & Stone B.A. (1972), Virology **50**, 791-798.
Nasser W. et al. (1988), Plant Mol. Biol. **11**, 529-538.
Parent J.G. & Asselin A. (1984), Can. J. Bot. **62**, 564-569.
Pegg G.F. & Young D.H. (1981), Physiol. Plant Pathol. **19**, 371-382.
Pegg G.F. & Young D.H. (1982), Physiol. Plant Pathol. **21**, 89-409.
Ross A.F. (1961), Virology **14**, 340-358.
Ross A.F. & Bozarth R.F. (1960), Phytopathology **50**, 652.
Sanger F. et al. (1977), Proc. Natl. Acad. Sci. USA **74**, 5463-5467.
Schlumbaum A. et al. (1986), Nature **324**, 365-367.
Sequeira L. (1983), Ann. Rev. Microbiol. **37**, 51-79.
Shinshi H. et al. (1987), Proc. Natl. Acad. Sci. USA **84**, 89-93.
Shinshi H. et al. (1988), Proc. Natl. Acad. Sci. USA **85**, 5541-5545.
Showalter A.M. et al. (1985), Proc. Natl. Acad. Sci. USA **82**, 6551-6555.
Skujins J.J. et al. (1965), Arch. Biochem. Biophys. **111**, 358-364.
Spanu P. et al. (1989), Planta **177**, 447-455.
Spurr (1973), Tob. Sci. **17**, 145-148.
Stuart I.M. et al. (1986), Plant Physiol. **80**, 310-314.
Swegle M. et al. (1989), Plant Mol. Biol. **12**, 403-412.
Toppan A. & Esquerré-Tugayé M.T. (1984), Plant Physiol. **75**, 1133-1138.
Van den Bulcke M. et al. (1989), Proc. Natl. Acad. Sci. USA **86**, 2673-2677.
Van den Elzen P.J.M. et al. (1985), Plant Mol. Biol. **5**, 299-302.
Van Loon L.C. (1975), Virology **67**, 566-575.
Van Loon L.C. & Antoniw J.F. (1982), N th. J. Plant Pathol. **88**, 237-256.
Vögeli U. et al. (1988), Planta **174**, 364-372.
Vög li-Lange R. et al. (1988), Plant Science **54**, 171-176.
Wargo P.M. (1975), Physiol. Plant Pathol. **5**, 99-105.

Wessels J.G.H. & Sietsma J.H. (1981), Encyclopedia of Plant Physiology, vol. 13B: Plant Carbohydrates II. pp. 352-394,
Tanner W., Lo wus F.A., eds. Springer, Berlin, Heidelberg, New York.
Woodward J.R. & Fincher G.B. (1982), Eur. J. Biochem. **121**, 663-669.
Woodward J.R. t al. (1982), FEBS Lett. **138**, 198-200.
Young D.H. & Pegg G.P. (1981), Physiol. Plant Pathol. **21**, 391-417.
Young D.H. & Pegg G.P. (1982), Physiol. Plant Pathol. **21**, 411-423.

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide, encoding protein
SEQUENCE LENGTH: 966

STRING TYPE: DOUBLE
TOPOLOGY: LINEAR
MOLECULE TYPE: cDNA

ORIGIN
ORGANISM: Petunia hybrida
CLONE: lambda-D1

PROPERTIES: Extracellular chitinase gene

```
                                     GAATTCCTAA TAATCGCGAA AAAA   24

 25 ATG AAG TTC TGG GGA TCA GTA TTG GCA TTG TCT TTT GTT GTG   66
    Met Lys Phe Trp Gly Ser Val Leu Ala Leu Ser Phe Val Val

 67 TTC TTG TTC CTA ACA GGA ACA CTG GCA CAA AAT GTT GGT TCT  108
    Phe Leu Phe Leu Thr Gly Thr Leu Ala Gln Asn Val Gly Ser

109 ATT GTG ACA AGC GAC TTA TTT GAC CAG ATG CTT AAA AAT AGG  150
    Ile Val Thr Ser Asp Leu Phe Asp Gln Met Leu Lys Asn Arg

151 AAT GAT GCT AGA TGT TTT GCC GTA CGG TTT TAC ACT TAC GAT  192
    Asn Asp Ala Arg Phe Phe Ala Val Arg Phe Tyr Thr Tyr Asp

193 GCC TTC ATA GCT GCT GCC AAT TCG TTC CCA GGT TTT GGA ACT  234
    Ala Phe Ile Ala Ala Ala Asn Ser Phe Pro Gly Phe Gly Thr

235 ACT GGT GAT GAT ACT GCC CGT AAG AAA GAA ATT GCT GCC TTT  276
    Thr Gly Asp Asp Thr Ala Arg Lys Lys Glu Ile Ala Ala Phe

277 TTC GGT CAA ACT TCT CAT GAA ACT ACT GGT GGT ACC TTA AGT  318
    Phe Gly Gln Thr Ser His Glu Thr Thr Gly Gly Thr Leu Ser

319 CCA GAT GGT CCA TAT GCA GGA GGA TAT TGC TTT CTT AGA GAA  360
    Pro Asp Gly Pro Tyr Gly Gly Gly Tyr Cys Phe Leu Arg Glu

361 GGC AAT CAA ATG GGA AAC GGA TAC TAT GGC AGA GGA CCC ATC  402
    Gly Asn Gln Met Gly Asn Gly Tyr Tyr Gly Arg Gly Pro Ile

403 CAA TTG ACA GGC CAA TCT AAC TAT GAC TTA GCT GGG AAA GCT  444
    Leu Leu Thr Gly Gln Ser Asn Tyr Asp Leu Ala Gly Lys Ala

445 ATT GAA CAA GAC TTA GTT AAC AAC CCT GAT TTA GTA GCA ACA  486
    Ile Glu Gln Asp Leu Val Asn Asn Pro Asp Leu Val Ala Thr

487 GAT GCT ACT GTA TCA TTC AAA ACA GCA ATA TGG TTC TGG ATG  528
    Asp Ala Thr Val Ser Phe Lys Thr Ala Ile Trp Phe Trp Met
```

SEQID 1

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

```
529 ACA CCA CAG GGT AAC AAG CCA TCT TGC CAC GAC GTT ATC ACC 570
    Thr Pro Gln Gly Asn Lys Pro Ser Cys His Asp Val Ile Thr

571 GGC CGA TGG ACG CCA TCA GCC GCC GAT ACA TCG GCG AAT CGT 612
    Gly Arg Trp Thr Pro Ser Ala Ala Asp Thr Ser Ala Asn Arg

613 GTA CCA GGT TAT GGT GTC ATT ACT AAC ATA ATT AAT GGT GGA 654
    Val Pro Gly Tyr Gly Val Ile Thr Asn Ile Ile Asn Gly Gly

655 ATT GAA TGT GGC AAA GGT CAG AAT GCA CGA GTG GAA GAT CGA 696
    Ile Glu Cys Gly Lys Gly Gln Asn Ala Arg Val Glu Asp Arg

697 ATT GGA TAT TAC AGG AGG AAT GTA AGT ATA ATG AAC GTG GCC 738
    Ile Gly Tyr Tyr Arg Arg Asn Val Ser Ile Met Asn Val Ala

739 CCT GGA GAC AAT TTG GAT TGT TAC AAC CAA AGG AAC TTT GCC 780
    Pro Gly Asp Asn Leu Asp Cys Tyr Asn Gln Arg Asn Phe Ala

781 GAA GTC                                                 786
    Glu Val

787 TAGGCTGGTC ACATTATGAG TGCAAATGTT ATGTAGTCAT GGAGATGACA  837

838 GTATACAACT TATATTTGAA TGTAATAAAT AAGGGATTCT CTATGCCCAT  887

888 TATGATAGAG TGAAATATAT TATTGTTTGT CTTCTTGGAA AGAAGTAGAA  937

938 CCAACAGTTC CTTTAAAAAA AAGGAATTC                         966
```

SEQID 1

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide, encoding protein
SEQUENCE LENGTH: 1252

STRING TYPE: DOUBLE
TOPOLOGY: LINEAR
MOLECULE TYPE: cDNA

ORIGIN
ORGANISM: NICOTIANA TABACUM Samsun NN.
CLONE: F1

PROPERTIES: Intracellular chitinase gene

```
                                                                  GGATCCAAC    9

 10 ATG AGG CTG TGC AAA TTC ACA GCT CTT TCT TCT CTA CTC TTT   51
    Met Arg Ile Cys Lys Phe Thr Ala Ile Ser Ser Ile Ile Phe

 52 TCT CTC CTA CTC CTC TCT GCC TCG GCA GAA CAA TGT GGT TCG   93
    Ser Ile Ile Ile Ile Ser Ala Ser Ala Glu Gln Cys Gly Ser

 94 CAG GCG GGA GGT GCG CGT TGT GCC TCG GGT CTC TGC TGC AGC  135
    Gln Ala Gly Gly Ala Arg Cys Ala Ser Gly Ile Cys Cys Ser

136 AAA TTT GGT TGG TGT GGT AAC ACC AAT GAC TAT TGT GGC CCT  177
    Lys Phe Gly Trp Cys Gly Asn Thr Asn Asp Tyr Cys Gly Pro

178 GGC AAT TGC CAG AGC CAG TGC CCT GGT GGT CCC ACA CCA CCC  219
    Gly Asn Cys Gln Ser Gln Cys Pro Gly Gly Pro Thr Pro Pro

220 GGT GGT GGG GAT CTC GGC AGT ATC ATC TCA AGT TCC ATG TTT  261
    Gly Gly Gly Asp Ile Gly Ser Ile Ile Ser Ser Ser Met Phe

262 GAT CAG ATG CTT AAG CAT CGC AAC GAT AAT GCA TGC CAA GGA  303
    Asp Gln Met Ile Lys His Arg Asn Asp Asn Ala Cys Gln Gly

304 AAG GGA TTC TAC AGT TAC AAT GCC TTT ATC AAT GCT GCT AGG  345
    Lys Gly Phe Tyr Ser Tyr Asn Ala Phe Ile Asn Ala Ala Arg

346 TCT TTT CCT GGC TTT GGT ACT AGT GGT GAT ACC ACT GCC CGT  387
    Ser Phe Pro Gly Phe Gly Thr Ser Gly Asp Thr Thr Ala Arg

388 AAA AGA GAA ATC GCG GCT TTC TTC GCC CAA ACC TCC CAT GAA  429
    Lys Arg Glu Ile Ala Ala Phe Phe Ala Gln Thr Ser His Glu

430 ACT ACA GGA GGA TGG GCA ACA GCA CCA GAT GGT CCA TAC GCG  471
    Thr Thr Gly Gly Trp Ala Thr Ala Pro Asp Gly Pro Tyr Ala

472 TGG GGT TAC TGC TGG CTT AGA GAA CAA TGT AGC CCC GGC GAC  513
    Trp Gly Tyr Cys Trp Ile Arg Glu Gln Cys Ser Pro Gly Asp

514 TAC TGT ACA CCA AGT GGT CAG TGG CCT TGT GCT CCT GGT CGG  555
    Tyr Cys Thr Pro Ser Gly Gln Trp Pro Cys Ala Pro Gly Arg
```

SEQID 2

MOGEN INT N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

556 AAA TAT TTC GGA CGA GGC CCC ATC CAA ATT TCA CAC AAC TAC 597
Lys Tyr Phe Gly Arg Gly Pro Ile Gln Ile Ser His Asn Tyr

598 AAC TAC GGA CCT TGT GGA AGA GCC ATA GGA GTG GAC CTC CTA 639
Asn Tyr Gly Pro Cys Gly Arg Ala Ile Gly Val Asp Ile Ile

640 AAC AAT CCT GAT TTA GTG GCC ACA GAT CCA GTA ATC TCA TTC 681
Asn Asn Pro Asp Ile Val Ala Thr Asp Pro Val Ile Ser Phe

682 AAG TCA GCT CTC TGG TTT TGG ATG ACT CCT CAA TCA CCA AAA 723
Lys Ser Ala Ile Trp Phe Trp Met Thr Pro Gln Ser Pro Lys

724 CCT TCT TGC CAC GAT GTC ATC ATT GGA AGA TGG CAA CCA TCG 765
Pro Ser Cys His Asp Val Ile Ile Gly Arg Trp Gln Pro Ser

766 TCT GCT GAC CGC GCA GCC AAT CGT CTC CCT GGA TTT GGT GTC 807
Ser Ala Asp Arg Ala Ala Asn Arg Ile Pro Gly Phe Gly Val

808 ATC ACG AAC ATC ATC AAT GGT GGC TTG GAA TGT GGT CGT GGC 849
Ile Thr Asn Ile Ile Asn Gly Gly Ile Glu Cys Gly Arg Gly

850 ACT GAC TCA AGG GTC CAG GAT CGC ATT GGG TTT TAC AGG AGG 891
Thr Asp Ser Arg Val Gln Asp Arg Ile Gly Phe Tyr Arg Arg

892 TAT TGC AGT ATT CTT GGT GTT AGT CCT GGT GAC AAT CTT GAT 933
Tyr Cys Ser Ile Ile Gly Val Ser Pro Gly Asp Asn Ile Asp

934 TGC GGA AAC CAG AGG TCT TTT GGA AAC GGA CTT TTA GTC GAT 975
Cys Gly Asn Gln Arg Ser Phe Gly Asn Gly Ile Ile Val Asp

976 ACT ATG
Thr Met

1082 TAATTTTATG GTCTGTTTTG TTGAATCCCT TTGCGACGCA GGGACCAGGG 1131

1132 GCTATGAATA AAGTTAATGT GTGAATTGTG TGATTGTCAT CTATGGGATC 1181

1182 GCGACTATAA TCGTTTATAA TAAACAAAGA CTTGTCCACA AAAAAAAAAA 1231

1232 GGAATTAATT CCCGGGGATC C

SEQID 2

MOGEN INT N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 3
SEQUENCE TYPE: Nucleotide, encoding protein
SEQUENCE LENGTH: 2020

STRING TYPE: DOUBLE
TOPOLOGY: LINEAR
MOLECULE TYPE: Genomic DNA

ORIGIN
ORGANISM: NICOTIANA TABACUM Samsun NN.
CLONE: gI9

PROPERTIES: Extracellular ß-1,3 glucanase gene

```
  1 CTTCTGCTTG TCTATATAAG AAGCAGCCTA ATGGTTCCTT AAACACACAA     50

 51 TTTCAGCTCA AGTGTTTCTT ACTCTCTCAT TTCCATTTTA GCT            93

 94 ATG ACT TTA TGC ATT AAA AAT GGC TTT CTT GCA GCT GCC CTT  135
    Met Thr Leu Cys Ile Lys Asn Gly Phe Leu Ala Ala Ala Leu

136 GTA CTT GTT GGG CTG TTA ATT TGC AGT ATC CAA ATG ATA G    175
    Val Leu Val Gly Leu Leu Ile Cys Ser Ile Gln Met Ile

176 GTCTCTCTCT CTCACACACA CACACTTTCT CTCATGATAC ATGTACATGC    225

226 ACCTTGTATG ATGCGGATCA ACTTATGTAC ACTAATAGCG TAAATAATTT    275

276 TTACAATATA TATTAGGATT AATATATTTT AACATGTTGT GTCAGGTAAT    325

326 CTACCTTATT TATTAAGTCA CTTATTATGA ATAGTTACTT ATAGTTACTT    375

376 CTGGGTGACC CGACACTATA ATGTTGGCTA GAGAAGAACT TAAATAGAGA    425

426 ATCATGGTTA GTGAGAATAT TCATTTATTC GACACCAACT TATTTGGGGA    475

476 CTGAAACTTC TTTGTAATAT ACTCTTTTTC TTACAATCCA G             516

517  GG GCA CAA TCT ATT GGA GTA TGC TAT GGA AAA CAT GCA AAC  557
    Gly Ala Gln Ser Ile Gly Val Cys Tyr Gly Lys His Ala Asn

558 AAT TTA CCA TCA GAC CAA GAT GTT ATA AAC CTA TAC AAT GCT  599
    Asn Leu Pro Ser Asp Gln Asp Val Ile Asn Leu Tyr Asn Ala

600 AAT GGC ATC AGA AAG ATG AGA ATC TAC AAT CCA GAT ACA AAT  641
    Asn Gly Ile Arg Lys Met Arg Ile Tyr Asn Pro Asp Thr Asn

642 GTC TTC AAC GCT CTC AGA GGA AGT AAC ATT GAG ATC ATT CTC  683
    Val Phe Asn Ala Leu Arg Gly Ser Asn Ile Glu Ile Ile Leu

684 GAC GTC CCA CTT CAA GAT CTT CAA TCC CTA ACT GAT CCT TCA  725
    Asp Val Pro Leu Gln Asp Leu Gln Ser Leu Thr Asp Pro Ser
```

SEQID 3

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

```
 726 AGA GCC AAT GGA TGG GTC CAA GAT AAC ATA ATA AAT CAT TTC  767
     Arg Ala Asn Gly Trp Val Gln Asp Asn Il  Ile Asn His Phe

 768 CCA GAT GTT AAA TTT AAA TAT ATA GCT GTT GGA AAT GAA GTC  809
     Pro Asp Val Lys Phe Lys Tyr Ile Ala Val Gly Asn Glu Val

 810 TCT CCC GGA AAT AAT GGT CAA TAT GCA CCA TTT GTT GCT CCT  851
     Ser Pro Gly Asn Asn Gly Gln Tyr Ala Pro Phe Val Ala Pro

 852 GCC ATG CAA AAT GTA TAT AAT GCA TTA GCA GCA GCA GGG TTG  893
     Ala Met Gln Asn Val Tyr Asn Ala Leu Ala Ala Ala Gly Leu

 894 CAA GAT CAA ATC AAG GTC TCA ACT GCA ACA TAT TCA GGG ATC  935
     Gln Asp Gln Ile Lys Val Ser Thr Ala Thr Tyr Ser Gly Ile

 936 TTA GCG AAT ACC TAC CCG CCC AAA GAT AGT ATT TTT CGA GGA  977
     Leu Ala Asn Thr Tyr Pro Pro Lys Asp Ser Ile Phe Arg Gly

 978 GAA TTC AAT AGT TTC ATT AAT CCC ATA ATC CAA TTT CTA GTA 1019
     Glu Phe Asn Ser Phe Ile Asn Pro Ile Ile Gln Phe Leu Val

1020 CAA CAT AAC CTT CCA CTC TTA GCC AAT GTC TAT CCT TAT TTT 1061
     Gln His Asn Leu Pro Leu Leu Ala Asn Val Tyr Pro Tyr Phe

1062 GGT CAC ATT TTC AAC ACT GCT GAT GTC CCA CTT TCT TAT GCT 1103
     Gly His Ile Phe Asn Thr Ala Asp Val Pro Leu Ser Tyr Ala

1104 TTG TTC ACA CAA CAA GAA GCA AAT CCT GCA GGA TAT CAA AAT 1145
     Leu Phe Thr Gln Gln Glu Ala Asn Pro Ala Gly Tyr Gln Asn

1146 CTT TTT GAT GCC CTT TTG GAT TCT ATG TAT TTT GCT GTA GAG 1187
     Leu Phe Asp Ala Leu Leu Asp Ser Met Tyr Phe Ala Val Glu

1188 AAA GCT GGA GGA CAA AAT GTG GAG ATT ATT GTA TCT GAA AGT 1229
     Lys Ala Gly Gly Gln Asn Val Glu Ile Ile Val Ser Glu Ser

1230 GGC TGG CCT TCT GAA GGA AAC TCT GCA GCA ACT ATT GAA AAT 1271
     Gly Trp Pro Ser Glu Gly Asn Ser Ala Ala Thr Ile Glu Asn

1272 GCT CAA ACT TAC TAT GAA AAT TTG ATT AAT CAT GTG AAA AGC 1313
     Ala Gln Thr Tyr Tyr Glu Asn Leu Ile Asn His Val Lys Ser

1314 GGG GCA GGA ACT CCA AAG AAA CCT GGA AAG GCT ATA GAA ACT 1355
     Gly Ala Gly Thr Pro Lys Lys Pro Gly Lys Ala Ile Glu Thr

1356 TAT TTA TTT GCC ATG TTT GAT GAA AAT AAT AAG GAA GGA GAT 1397
     Tyr Leu Phe Ala Met Phe Asp Glu Asn Asn Lys Glu Gly Asp

1398 ATC ACA GAG AAA CAC TTT GGA CTC TTT TCT CCT GAT CAG AGG 1439
     Ile Thr Glu Lys His Phe Gly Leu Phe Ser Pro Asp Gln Arg

1440 GCA AAA TAT CAA CTC AAT TTC AAT                         1463
     Ala Lys Tyr Gln Leu Asn Phe Asn
```

SEQID 3

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

```
1464 TAATTAATGC ATGGTAACAT TTATTGATAT ATATAGTGAT ATGAGTAATA 1513
1514 AGGAGAAGTA GAACTGCTAT GTTTTTCTCT TCAATTGAAA ATGTAACTCT 1563
1564 GGTTTCACTT TGATATTTAT ATGACATATT TATTGAGATC TCGTCTTTTG 1613
1614 TTTTAAATTC TTGCCTTCTA TTGGCAAATA TCTGCGTAAT TTTCATTTGT 1663
1664 TTTAAAAATT ACTAAGCCTC AAAAGAGTGA CTACCAATAT ATTCTTGATT 1713
1714 ATTAATATTC CCCGTGCTTG GGGGACCGGG TGAGGTGGGG GGTGGGGGGG 1763
1764 ATGACGAAAA AAGTTAATGA AAAACCGGTT TGCATTGGAT GCTCTTTTTA 1813
1814 ACCTCCCCAA AATATGATGG TTTTGTTGTC TTGGAGAGTG TTTAAGCTAC 1863
1864 TTCTTCTCAA GAATTTTCTT GGTCAGTTCT TAACGTAATT GCTTTTAATT 1913
1914 TCTTAATTAT CGGTAACCCT TCGAAACAAA AGGAAAATTA AGCTAGGAGA 1963
1964 TGACTCGTAT TCATAATGTT TTACCTTGGA TCAACCCCGC CTTTATATTT 2013
2014 CATACGA                                                2020
```

SEQID 3

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 4
SEQUENCE TYPE: Nucleotide, encoding protein
SEQUENCE LENGTH: 2509

STRING TYPE: DOUBLE
TOPOLOGY: LINEAR
MOLECULE TYPE: Genomic DNA

ORIGIN
ORGANISM: NICOTIANA TABACUM Samsun NN.
CLONE: gGLB50

PROPERTIES: Intracellular ß-1,3 glucanase gene

```
  1 AATATAAATA GCTCGTTGTT CATCTTAATT CTCCCAACAA GTCTTCCCAT   50

 51 CATGTCTACC TCACATAAAC ATAATACTCC TCAA                   84

 85 ATG GCT GCT ATC ACA CTC CTA GGA TTA CTA CTT GTT GCC AGC 126
    Met Ala Ala Ile Thr Leu Leu Gly Leu Leu Leu Val Ala Ser

127 AGC ATT GAC ATA GCA G                                   142
    Ser Ile Asp Ile Ala

143 GTTTCTGGTC AAATATTTGA ACTTCCCAGC CAAAAATATT GTCTTATAAT 192

193 TTTGTGTGCG CAAAATTTTA ATTTAGTTGA TAGTTATTTG CTTATTTTTC 242

243 TTTTCAAATT GCTTGTGTTT TTTTCTCAAA TTAACTTGCA CCGTATTCAT 292

293 TTAGCGATAG TTATTTGCTC TATTTTGTGT AACACTCACT CACAAACTTT 342

393 TCAATTTGAG GGGAGGACAG TGAATCTAAG ATTGAAATTT ATGAGTTTAA 392

393 TTAGACTAAT TCCCATTTGA TTTATTGGCT AGAAGTCAAT TATTTGCATA 442

443 GTGAGTCTTT TAACACACAG ATTTGAGTTA AAGCTACTAC GTTCGTATTA 492

493 ACCCATAACA TATACACCTT CTGTTCTAAT TTCTTTGACA CTTTTTGTTA 542

543 GTTTGTTCCA AAAAGGACGG ACATATTTGA TATTTGAGAA TACTTTACCT 592

593 TAACCTTAAT AGAATTTTTT ATGACATCAC ATATATTATG GAATATATAC 642

643 GACCATAATT TTCAAATATC TTATAGTCGT ACAAATATTA TAGCATGTTT 692

693 AATACCACAA CTTTCAAATT CTTCTTTTCC TTAAAAACAA AATATGTCAC 742

743 ATAAATTAAA ATAGAGGAAG TATACTACAT CAATCAGCCC CTAGTGGAGG 792

793 GGACCTACTG TAAGTTTTTA AGTTTTCAAG AATTCAGTAA TTGATTAGGA 842
```

SEQID 4

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

```
843 GCCCGTCTGG ACATAAAAAA AAATTCCTTT TTTTCCAAAA AATGCCCACT  892

893 AAATTTCTAA CACTATTTTG TAATTCTTAT TGAGCAG                929

930  GG GCT CAA TCG ATA GGT GTT TGC TAT GGA ATG CTA GGC AAC  970
    Gly Ala Gln Ser Ile Gly Val Cys Tyr Gly Met Leu Gly Asn

971 AAC TTG CCA AAT CAT TGG GAA GTT ATA CAG CTC TAC AAG TCA 1012
    Asn Leu Pro Asn His Trp Glu Val Ile Gln Leu Tyr Lys Ser

1013 AGA AAC ATA GGA AGA CTG AGG CTT TAT GAT CCA AAT CAT GGA 1054
     Arg Asn Ile Gly Arg Leu Arg Leu Tyr Asp Pro Asn His Gly

1055 GCT TTA CAA GCA TTA AAA GGC TCA AAT ATT GAA GTT ATG TTA 1096
     Ala Leu Gln Ala Leu Lys Gly Ser Asn Ile Glu Val Met Leu

1097 GGA CTT CCC AAT TCA GAT GTG AAG CAC ATT GCT TCC GGA ATG 1138
     Gly Leu Pro Asn Ser Asp Val Lys His Ile Ala Ser Gly Met

1139 GAA CAT GCA AGA TGG TGG GTA CAG AAA AAT GTT AAA GAT TTC 1180
     Glu His Ala Arg Trp Trp Val Gln Lys Asn Val Lys Asp Phe

1181 TGG CCA GAT GTT AAG ATT AAG TAT ATT GCT GTT GGG AAT GAA 1222
     Trp Pro Asp Val Lys Ile Lys Tyr Ile Ala Val Gly Asn Glu

1223 ATC AGC CCT GTC ACT GGC ACA TCT TAC CTA ACC TCA TTT CTT 1264
     Ile Ser Pro Val Thr Gly Thr Ser Tyr Leu Thr Ser Phe Leu

1265 ACT CCT GCT ATG GTA AAT ATT TAC AAA GCA ATT GGT GAA GCT 1306
     Thr Pro Ala Met Val Asn Ile Tyr Lys Ala Ile Gly Glu Ala

1307 GGT TTG GGA AAC AAC ATC AAG GTC TCA ACT TCT GTA GAC ATG 1348
     Gly Leu Gly Asn Asn Ile Lys Val Ser Thr Ser Val Asp Met

1349 ACC TTG ATT GGA AAC TCT TAT CCA CCA TCA CAG GGT TCG TTT 1390
     Thr Leu Ile Gly Asn Ser Tyr Pro Pro Ser Gln Gly Ser Phe

1391 AGG AAC GAT GCT AGG TGG TTT GTT GAT GCC ATT GTT GGC TTC 1432
     Arg Asn Asp Ala Arg Trp Phe Val Asp Ala Ile Val Gly Phe

1433 TTA AGG GAC ACA CGT GCA CCT TTA CTC GTT AAC ATT TAC CCC 1474
     Leu Arg Asp Thr Arg Ala Pro Leu Leu Val Asn Ile Tyr Pro

1475 TAT TTC AGT TAT TCT GGT AAT CCA GGC CAG ATT TCT CTC CCC 1516
     Tyr Phe Ser Tyr Ser Gly Asn Pro Gly Gln Ile Ser Leu Pro

1517 TAT TCT CTT TTT ACA GCA CCA AAT GTG GTG GTA CAA GAT GGT 1558
     Tyr Ser Leu Phe Thr Ala Pro Asn Val Val Val Gln Asp Gly

1559 TCC CGC CAA TAT AGG AAC TTA TTT GAT GCA ATG CTG GAT TCT 1600
     Ser Arg Gln Tyr Arg Asn Leu Phe Asp Ala Met Leu Asp Ser
```

SEQID 4

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

```
1601 GTG TAT GCT GCC CTC GAG CGA TCA GGA GGG GCA TCT GTA GGG 1642
     Val Tyr Ala Ala Leu Glu Arg Ser Gly Gly Ala Ser Val Gly

1643 ATT GTT GTG TCC GAG AGT GGC TGG CCA TCT GCT GGT GCA TTT 1684
     Ile Val Val Ser Glu Ser Gly Trp Pro Ser Ala Gly Ala Phe

1685 GGA GCC ACA TAT GAC AAT GCA GCA ACT TAC TTG AGG AAC TTA 1726
     Gly Ala Thr Tyr Asp Asn Ala Ala Thr Tyr Leu Arg Asn Leu

1727 ATT CAA CAC GCT AAA GAG GGT AGC CCA AGA AAG CCT GGA CCT 1768
     Ile Gln His Ala Lys Glu Gly Ser Pro Arg Lys Pro Gly Pro

1769 ATT GAG ACC TAT ATA TTT GCC ATG TTT GAT GAG AAC AAC AAG 1810
     Ile Glu Thr Tyr Ile Phe Ala Met Phe Asp Glu Asn Asn Lys

1811 AAC CCT GAA CTG GAG AAA CAT TTT GGA TTG TTT TCC CCC AAC 1852
     Asn Pro Glu Leu Glu Lys His Phe Gly Leu Phe Ser Pro Asn

1853 AAG CAG CCC AAA TAT AAT ATC AAC TTT GGG GTC TCT GGT GGA 1894
     Lys Gln Pro Lys Tyr Asn Ile Asn Phe Gly Val Ser Gly Gly

1895 GTT TGG GAC AGT TCA GTT GAA ACT AAT GCT ACT GCT TCT CTC 1936
     Val Trp Asp Ser Ser Val Glu Thr Asn Ala Thr Ala Ser Leu

1937 GTA AGT GAG ATG                                         1948
     Val Ser Glu Met

1949 TGAGCTGATG AGACACTTGA AATCTCTTTA CATACGTATT CCTTGGATGG  1998

1999 AAAACCTAGT AAAAACAAGA GAAATTTTTT CTTTATGCAA GATACTAAAT  2048

2049 AACATTGCAT GTCTCTGTAA GTCCTCATGG ATTGTTATCC AGTGACGATG  2098

2099 CAACTCTGAG TGGTTTTAAA TTCCTTTTCT TTGTGATATT GGTAATTTGG  2148

2149 CAAGAAACTT TCTGTAAGTT TGTGAATTTC ATGCATCATT AATTATACAT  2198

2199 CAGTTCCATG TTTGATCAGA TTGGGATTTG GTAACTTCAA TGTTAGTATT  2248

2249 ATAATTAGTG TCTTTATCAT TGACTATCAA TTAATCTTTA TTTGGCAAGG  2298

2299 CTTGATATAT TTGAGTTACT CTTAGGTATT TGCAAGCAAC TGATCTTTCT  2348

2349 TTTATCCCGT TTCTGGCTTA AACCTCATTA GAAATATATT ATAATGTCAC  2398

2399 CTACTCTGTG GTTTAAGACA TTCCCTTACA TTATAAGGTA TTTCACGTCG  2448

2449 TATCAGGTCG AAAAAAATAA TGGTACGCTC TTTCTTATCA CAAATTTCTC  2498

2499 TAACTTCTAG A                                             2509
```

SEQID 4

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 5
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 50

STRANDEDNESS: DOUBLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Polylinker sequence

1 GGAATTCTGG TACCTCCCGG GAGGATCCAT CTAGAGCTCG AGTAAGCTTC 50

SEQID 5

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 6
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 24

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 AGCTTGGATC CGTCGACGGA TCCT 24

SEQID 6

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 7
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 24

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 AATTAGGATC CGTCGACGGA TCCA 24

SEQID 7

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 8
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 21

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 GATCCAACAT GAGGCTGTGC A 21

SEQID 8

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 9
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 21

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 AATTTGCACA GCCTCATGTT G 21

SEQID 9

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 10
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 10

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 AATTGAGCTC 10

SEQID 10

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

SEQ ID NO: 11
SEQUENCE TYPE: Nucl otide
SEQUENCE LENGTH: 6

STRANDEDNESS: SINGLE
TOPOLOGY: LINEAR
MOLECULE TYPE: SYNTHETIC FRAGMENT

ORIGIN
ORGANISM: -
CLONE: -

PROPERTIES: Adaptor sequence for cloning

1 CAATTC 6

SEQID 11

MOGEN INT. N.V.
RIJKSUNIVERSITEIT
TE LEIDEN

Claims

1. A plant which exhibits, as a result of genetic manipulation of the said plant or an ancestor thereof, through the use of one or more recombinant polynucleotides, a relative overexpression of an intracellular chitinase gene, in at least one of the tissues.

2. The plant according to Claim 1, in which the intracellular chitinase is targeted to the apoplast, due to modification of the gene encoding it.

3. The plant according to Claim 2, in which the gene encoding said intracellular chitinase gene is modified by creating a translation stopcodon in the coding region at the 3'-end of the gene.

4. The plant according to Claim 3, in which the creation of the translation stopcodon results in deletion of between 3 and 10 C-terminal amino acids.

5. A plant which exhibits, as a result of the genetic manipulation of the said plant, or an ancestor, or any plant part regenerated to produce the said plant, through the use of one or more recombinant polynucleotides, a relative overexpression of a chitinase gene and a β-1,3-glucanase gene in at least one of the tissues.

6. The plant according to any of the Claims 1 to 4, which further exhibits the relative overexpression of at least one gene encoding an enzyme selected from the group consisting of extracellular chitinases, intracellular β-1,3-glucanases, and extracellular β-1,3-glucanases.

7. The plant according to any of the Claims 1-4, which further exhibits the relative overexpression of at least an extracellular chitinase gene and an intracellular β-1,3-glucanase gene.

8. The plant according to any of the Claims 1-4, which further exhibits the relative overexpression of an extracellular chitinase gene, an intracellular β-1,3-glucanase gene and an extracellular β-1,3-glucanase gene.

9. The plant according to any of the Claims 6 to 8, in which the intracellular β-1,3-glucanase is targeted to the apoplast, due to modification of the gene encoding it.

10. The plant according to Claim 9, in which the gene encoding the intracellular β-1,3-glucanase gene is modified by creating a translation stopcodon in the coding region at the 3'-end of the gene.

11. The plant according to Claim 10, in which the creation of the translation stopcodon results in deletion of between 3 and 25 C-terminal amino acids of the intracellular β-1,3-glucanase.

12. The plant according to any of the Claims 1 to 11, in which the newly introduced genes are under the control of the CaMV 35S promoter.

13. A recombinant polynucleotide, comprising genetic information for the relative overexpression of an intracellular chitinase gene, essentially comprising,
    a) a promoter that is functional in plants,
    b) a gene encoding the intracellular chitinase, under the control of the said promoter, and
    c) a terminator operably linked to the said gene, and
    d) a gene encoding a selectable or screenable trait, operably linked to regulatory sequences for proper expression.

14. A recombinant polynucl otide according to Claim 13, in which the gen ncoding the intracellular chitinase is modified by creating a translation stopcodon in the coding region at th 3'-end of th gene.

15. A r combinant polynucleotide according to Claim 14, in which th stopcodon results in d letion of

between 3 and 10 C-terminal amino acids of th intracellular chitinase.

16. A recombinant polynucleotide according to any of the Claims 13 to 15, additionally comprising the genetic information for the relative overexpression of at least one gene encoding an enzyme selected from the group consisting of extracellular chitinases, intracellular β-1,3-glucanases and xtracellular β-1,3-glucanases, said genetic information essentially comprising,
   e) a promoter that is functional in plants,
   f) the said gene encoding one of the said enzymes, under the control of the said promoter, and
   g) a terminator, operably linked to the said gene.

17. A recombinant polynucleotide according to Claim 16, additionally comprising the genetic information for the relative overexpression of a gene encoding an extracellular chitinase, in which f) is a gene encoding an extracellular chitinase.

18. A recombinant polynucleotide according to Claim 16, additionally comprising the genetic information for the relative overexpression of a gene encoding an intracellular β-1,3-glucanase, in which f) is a gene encoding an intracellular β-1,3-glucanase.

19. A recombinant polynucleotide according to Claim 16, additionally comprising the genetic information for the relative overexpression of a gene encoding an extracellular β-1,3-glucanase, in which f) is a gene encoding an extracellular β-1,3-glucanase.

20. A recombinant polynucleotide, comprising the genetic information for the relative overexpression of an intracellular β-1,3-glucanase gene, essentially comprising,
   a) a promoter that is functional in plants,
   b) a gene encoding the intracellular β-1,3-glucanase, under the control of the said promoter,
   c) a terminator, operably linked to the said gene, and
   d) a gene encoding a selectable or screenable trait, operably linked to regulatory sequences for proper expression.

21. A recombinant polynucleotide, according to Claim 16, 18, or 20, in which the gene encoding the intracellular β-1,3-glucanase is modified by creating a translation stopcodon in the coding region at the 3'-end of the gene.

22. A recombinant polynucleotide according to Claim 21, in which the stopcodon results in deletion of between 3 and 25 C-terminal amino acids of the intracellular β-1,3-glucanase.

23. A recombinant polynucleotide according to any of the Claims 21 or 22, additionally comprising the genetic information for the relative overexpression of at least a gene encoding an extracellular β-1,3-glucanase, essentially comprising,
   e) a promoter that is functional in plants,
   f) a gene encoding the extracellular β-1,3-glucanase, under the control of the said promoter, and
   g) a terminator, operably linked to the said gene.

24. A cloning or transformation vector comprising the recombinant polynucleotides of any of the Claims 13 to 23.

25. A plasmid selected from the group consisting of pMOG200, pMOG212, pMOG289 and pMOG512, as well as derivatives thereof.

26. Bacteria harboring at least a plasmid according to Claim 25.

27. A process for obtaining fungal resistant plants by introducing into the genome of the said plants, or their ancestors, or any plant part that can be regenerated to produce the said plant, a recombinant polynucleotide of any one of the Claims 13 to 23.

FIG., 1.

```
                                   M  K  F  W  G  S  V  L  A  L  S  F
GAATTCCTAATAATCGCGAAAAAAATGAAGTTCTGGGGATCAGTATTGGCATTGTCTTTT
       10                  30                  50

V  V  F  L  F  L  T  G  T  L  A  Q  N  V  G  S  I  V  T  S
GTTGTGTTCTTGTTCCTAACAGGAACACTGGCACAAAATGTTGGTTCTATTGTGACAAGC
       70                  90                  110

D  L  F  D  Q  M  L  K  N  R  N  D  A  R  C  F  A  V  R  F
GACTTATTTGACCAGATGCTTAAAAATAGGAATGATGCTAGATGTTTTGCCGTACGGTTT
       130                 150                 170

Y  T  Y  D  A  F  I  A  A  A  N  S  F  P  G  F  G  T  T  G
TACACTTACGATGCCTTCATAGCTGCTGCCAATTCGTTCCCAGGTTTTGGAACTACTGGT
       190                 210                 230

D  D  T  A  R  K  K  E  I  A  A  F  F  G  Q  T  S  H  E  T
GATGATACTGCCCGTAAGAAAGAAATTGCTGCCTTTTTCGGTCAAACTTCTCATGAAACT
       250                 270                 290

T  G  G  T  L  S  P  D  G  P  Y  A  G  G  Y  C  F  L  R  E
ACTGGTGGTACCTTAAGTCCAGATGGTCCATATGCAGGAGGATATTGCTTTCTTAGAGAA
       310                 330                 350

G  N  Q  M  G  N  G  Y  Y  G  R  G  P  I  Q  L  T  G  Q  S
GGCAATCAAATGGGAAACGGATACTATGGCAGAGGACCCATCCAATTGACAGGCCAATCT
       370                 390                 410

N  Y  D  L  A  G  K  A  I  E  Q  D  L  V  N  N  P  D  L  V
AACTATGACTTAGCTGGGAAAGCTATTGAACAAGACTTAGTTAACAACCCTGATTTAGTA
       430                 450                 470

A  T  D  A  T  V  S  F  K  T  A  I  W  F  W  M  T  P  Q  G
GCAACAGATGCTACTGTATCATTCAAAACAGCAATATGGTTCTGGATGACACCACAGGGT
       490                 510                 530

N  K  P  S  C  H  D  V  I  T  G  R  W  T  P  S  A  A  D  T
AACAAGCCATCTTGCCACGACGTTATCACCGGCCGATGGACGCCATCAGCCGCCGATACA
       550                 570                 590

S  A  N  R  V  P  G  Y  G  V  I  T  N  I  I  N  G  G  I  E
TCGGCGAATCGTGTACCAGGTTATGGTGTCATTACTAACATAATTAATGGTGGAATTGAA
       610                 630                 650

C  G  K  G  Q  N  A  R  V  E  D  R  I  G  Y  Y  R  R  N  V
TGTGGCAAAGGTCAGAATGCACGAGTGGAAGATCGAATTGGATATTACAGGAGGAATGTA
       670                 690                 710

S  I  M  N  V  A  P  G  D  N  L  D  C  Y  N  Q  R  N  F  A
AGTATAATGAACGTGGCCCCTGGAGACAATTTGGATTGTTACAACCAAAGGAACTTTGCC
       730                 750                 770
E  V  *
GAAGTCTAGGCTGGTCACATTATGAGTGCAAATGTTATGTAGTCATGGAGATGACAGTAT
       790                 810                 830
ACAACTTATATTTGAATGTAATAAATAAGGGATTCTCTATGCCCATTTATGATAGAGTGA
       850                 870                 890
AATATATTATTGTTTGTCTTCTTGGAAAGAAGTAGAACCAACAGTTCCTTTAAAAAAAAGGAATTC
       910                                     950
```

FIG. 2

```
      M  R  L  C  K  F  T  A  L  S  S  L  L  F  S  L  L
GGATCCAACATGAGGCTGTGCAAATTCACAGCTCTTTCTTCTCTACTCTTTTCTCTCCTA
    10                  30                  50

L  L  S  A  S  A  E  Q  C  G  S  Q  A  G  G  A  R  C  A  S
CTCCTCTCTGCCTCGGCAGAACAATGTGGTTCGCAGGCGGGAGGTGCGCGTTGTGCCTCG
       70                  90                  110

G  L  C  C  S  K  F  G  W  C  G  N  T  N  D  Y  C  G  P  G
GGTCTCTGCTGCAGCAAATTTGGTTGGTGTGGTAACACCAATGACTATTGTGGCCCTGGC
       130                 150                 170

N  C  Q  S  Q  C  P  G  G  P  T  P  P  G  G  G  D  L  G  S
AATTGCCAGAGCCAGTGCCCTGGTGGTCCCACACCACCCGGTGGTGGGGATCTCGGCAGT
       190                 210                 230

I  I  S  S  S  M  F  D  Q  M  L  K  H  R  N  D  N  A  C  Q
ATCATCTCAAGTTCCATGTTTGATCAGATGCTTAAGCATCGCAACGATAATGCATGCCAA
       250                 270                 290

G  K  G  F  Y  S  Y  N  A  F  I  N  A  A  R  S  F  P  G  F
GGAAAGGGATTCTACAGTTACAATGCCTTTATCAATGCTGCTAGGTCTTTTCCTGGCTTT
       310                 330                 350

G  T  S  G  D  T  T  A  R  K  R  E  I  A  A  F  F  A  Q  T
GGTACTAGTGGTGATACCACTGCCCGTAAAAGAGAAATCGCGGCTTTCTTCGCCCAAACC
       370                 390                 410

S  H  E  T  T  G  G  W  A  T  A  P  D  G  P  Y  A  W  G  Y
TCCCATGAAACTACAGGAGGATGGGCAACAGCACCAGATGGTCCATACGCGTGGGGTTAC
       430                 450                 470

C  W  L  R  E  Q  C  S  P  G  D  Y  C  T  P  S  G  Q  W  P
TGCTGGCTTAGAGAACAATGTAGCCCCGGCGACTACTGTACACCAAGTGGTCAGTGGCCT
       490                 510                 530

C  A  P  G  R  K  Y  F  G  R  G  P  I  Q  I  S  H  N  Y  N
TGTGCTCCTGGTCGGAAATATTTCGGACGAGGCCCCATCCAAATTTCACACAACTACAAC
       550                 570                 590

Y  G  P  C  G  R  A  I  G  V  D  L  L  N  N  P  D  L  V  A
TACGGACCTTGTGGAAGAGCCATAGGAGTGGACCTCCTAAACAATCCTGATTTAGTGGCC
       610                 630                 650

T  D  P  V  I  S  F  K  S  A  L  W  F  W  M  T  P  Q  S  P
ACAGATCCAGTAATCTCATTCAAGTCAGCTCTCTGGTTTTGGATGACTCCTCAATCACCA
       670                 690                 710

K  P  S  C  H  D  V  I  I  G  R  W  Q  P  S  S  A  D  R  A
AAACCTTCTTGCCACGATGTCATCATTGGAAGATGGCAACCATCGTCTGCTGACCGCGCA
       730                 750                 770

A  N  R  L  P  G  F  G  V  I  T  N  I  I  N  G  G  L  E  C
GCCAATCGTCTCCCTGGATTTGGTGTCATCACGAACATCATCAATGGTGGCTTGGAATGT
       790                 810                 830
```

FIG 2 (vervolg)

```
G  R  G  T  D  S  R  V  Q  D  R  I  G  F  Y  R  R  Y  C  S
GGTCGTGGCACTGACTCAAGGGTCCAGGATCGCATTGGGTTTTACAGGAGGTATTGCAGT
        850                 870                 890

I  L  G  V  S  P  G  D  N  L  D  C  G  N  Q  R  S  F  G  N
ATTCTTGGTGTTAGTCCTGGTGACAATCTTGATTGCGGAAACCAGAGGTCTTTTGGAAAC
        910                 930                 950

G  L  L  V  D  T  M  *
GGACTTTTAGTCGATACTATGTAATTTTATGGTCTGTTTTGTTGAATCCCTTTGCGACGC
        970                 990                1010

AGGGACCAGGGGCTATGAATAAAGTTAATGTGTGAATTGTGTGATTGTCATCTATGGGAT
       1030                1050                1070

CGCGACTATAATCGTTTATAATAAACAAAGACTTGTCCACAAAAAAAAAAAGGAATTAAT
       1090                1110                1130

TCCCGGGGATCC
       1150
```

FIG 3

```
CTTCTGCTTGTCTATATAAGAAGCAGCCTAATGGTTCCTTAAACACACAATTTCAGCTCA
        10                          30                          50
                                   M   T   L   C   I   K   N   G   F
AGTGTTTCTTACTCTCTCATTTCCATTTTAGCTATGACTTTATGCATTAAAAATGGCTTT
        70                          90                          110
L   A   A   A   L   V   L   V   G   L   L   I   C   S   I   Q   M   I   G
CTTGCAGCTGCCCTTGTACTTGTTGGGCTGTTAATTTGCAGTATCCAAATGATAGGTCTC
        130                         150                         170
     intron
TCTCTCTCACACACACACACTTTCTCTCATGATACATGTACATGCACCTTGTATGATGCG
        190                         210                         230
GATCAACTTATGTACACTAATAGCGTAAATAATTTTTACAATATATATTAGGATTAATAT
        250                         270                         290
ATTTTAACATGTTGTGTCAGGTAATCTACCTTATTTATTAAGTCACTTATTATGAATAGT
        310                         330                         350
TACTTATAGTTACTTCTGGGTGACCCGACACTATAATGTTGGCTAGAGAAGAACTTAAAT
        370                         390                         410
AGAGAATCATGGTTAGTGAGAATATTCATTTATTCGACACCAACTTATTTGGGGACTGAA
        430                         450                         470
                           intron                 A   Q   S   I   G   V   C   Y
ACTTCTTTGTAATATACTCTTTTTCTTACAATCCAGGGGCACAATCTATTGGAGTATGCT
        490                         510                         530
 G   K   H   A   N   N   L   P   S   D   Q   D   V   I   N   L   Y   N   A   N
ATGGAAAACATGCAAACAATTTACCATCAGACCAAGATGTTATAAACCTATACAATGCTA
        550                         570                         590
 G   I   R   K   M   R   I   Y   N   P   D   T   N   V   F   N   A   L   R   G
ATGGCATCAGAAAGATGAGAATCTACAATCCAGATACAAATGTCTTCAACGCTCTCAGAG
        610                         630                         650
 S   N   I   E   I   I   L   D   V   P   L   Q   D   L   Q   S   L   T   D   P
GAAGTAACATTGAGATCATTCTCGACGTCCCACTTCAAGATCTTCAATCCCTAACTGATC
        670                         690                         710
 S   R   A   N   G   W   V   Q   D   N   I   I   N   H   F   P   D   V   K   F
CTTCAAGAGCCAATGGATGGGTCCAAGATAACATAATAAATCATTTCCCAGATGTTAAAT
        730                         750                         770
 K   Y   I   A   V   G   N   E   V   S   P   G   N   N   G   Q   Y   A   P   F
TTAAATATATAGCTGTTGGAAATGAAGTCTCTCCCGGAAATAATGGTCAATATGCACCAT
        790                         810                         830
 V   A   P   A   M   Q   N   V   Y   N   A   L   A   A   A   G   L   Q   D   Q
TTGTTGCTCCTGCCATGCAAAATGTATATAATGCATTAGCAGCAGCAGGGTTGCAAGATC
        850                         870                         890
 I   K   V   S   T   A   T   Y   S   G   I   L   A   N   T   Y   P   P   K   D
AAATCAAGGTCTCAACTGCAACATATTCAGGGATCTTAGCGAATACCTACCCGCCCAAAG
        910                         930                         950
 S   I   F   R   G   E   F   N   S   F   I   N   P   I   I   Q   F   L   V   Q
ATAGTATTTTTCGAGGAGAATTCAATAGTTTCATTAATCCCATAATCCAATTTCTAGTAC
        970                         990                         1010
 H   N   L   P   L   L   A   N   V   Y   P   Y   F   G   H   I   F   N   T   A
AACATAACCTTCCACTCTTAGCCAATGTCTATCCTTATTTTGGTCACATTTTCAACACTG
       1030                         1050                        1070
 D   V   P   L   S   Y   A   L   F   T   Q   Q   E   A   N   P   A   G   Y   Q
CTGATGTCCCACTTTCTTATGCTTTGTTCACACAACAAGAAGCAAATCCTGCAGGATATC
       1090                         1110                        1130
 N   L   F   D   A   L   L   D   S   M   Y   F   A   V   E   K   A   G   G   Q
AAAATCTTTTTGATGCCCTTTTGGATTCTATGTATTTTGCTGTAGAGAAAGCTGGAGGAC
       1150                         1170                        1190
 N   V   E   I   I   V   S   E   S   G   W   P   S   E   G   N   S   A   A   T
```

FIG 3 (vervolg)

```
AAAATGTGGAGATTATTGTATCTGAAAGTGGCTGGCCTTCTGAAGGAAACTCTGCAGCAA
        1210                      1230                      1250
 I   E   N   A   Q   T   Y   Y   E   N   L   I   N   H   V   K   S   G   A   G
CTATTGAAAATGCTCAAACTTACTATGAAAATTTGATTAATCATGTGAAAAGCGGGGCAG
        1270                      1290                      1310
 T   P   K   K   P   G   K   A   I   E   T   Y   L   F   A   M   F   D   E   N
GAACTCCAAAGAAACCTGGAAAGGCTATAGAAACTTATTTATTTGCCATGTTTGATGAAA
        1330                      1350                      1370
 N   K   E   G   D   I   T   E   K   H   F   G   L   F   S   P   D   Q   R   A
ATAATAAGGAAGGAGATATCACAGAGAAACACTTTGGACTCTTTTCTCCTGATCAGAGGG
        1390                      1410                      1430
 K   Y   Q   L   N   F   N
CAAAATATCAACTCAATTTCAATTAATTAATGCATGGTAACATTTATTGATATATATAGT
        1450                      1470                      1490
GATATGAGTAATAAGGAGAAGTAGAACTGCTATGTTTTTCTCTTCAATTGAAAATGTAAC
        1510                      1530                      1550
TCTGGTTTCACTTTGATATTTATATGACATATTTATTGAGATCTCGTCTTTTGTTTTAAA
        1570                      1590                      1610
TTCTTGCCTTCTATTGGCAAATATCTGCGTAATTTTCATTTGTTTTAAAAATTACTAAGC
        1630                      1650                      1670
CTCAAAAGAGTGACTACCAATATATTCTTGATTATTAATATTCCCCGTGCTTGGGGGACC
        1690                      1710                      1730
GGGTGAGGTGGGGGGTGGGGGGGATGACGAAAAAAGTTAATGAAAAACCGGTTTGCATTG
        1750                      1770                      1790
GATGCTCTTTTTAACCTCCCCAAAATATGATGGTTTTGTTGTCTTGGAGAGTGTTTAAGC
        1810                      1830                      1850
TACTTCTTCTCAAGAATTTTCTTGGTCAGTTCTTAACGTAATTGCTTTTAATTTCTTAAT
        1870                      1890                      1910
TATCGGTAACCCTTCGAAACAAAAGGAAAATTAAGCTAGGAGATGACTCGTATTCATAAT
        1930                      1950                      1970
GTTTTACCTTGGATCAACCCCGCCTTTATATTTCATACGA
        1990                      2010
```

FIG 4

```
AATATAAATAGCTCGTTGTTCATCTTAATTCTCCCAACAAGTCTTCCCATCATGTCTACC
        10                  30                  50
                         M  A  A  I  T  L  L  G  L  L  L  V
TCACATAAACATAATACTCCTCAAATGGCTGCTATCACACTCCTAGGATTACTACTTGTT
        70                  90                 110

A  S  S  I  D  I  A  G---------->intron
GCCAGCAGCATTGACATAGCAGGTTTCTGGTCAAATATTTGAACTTCCCAGCCAAAAATA
       130                 150                 170
TTGTCTTATAATTTTGTGTGCGCAAAATTTTAATTTAGTTGATAGTTATTTGCTTATTTT
       190                 210                 230
TCTTTTCAAATTGCTTGTGTTTTTTTCTCAAATTAACTTGCACCGTATTCATTTAGCGAT
       250                 270                 290
AGTTATTTGCTCTATTTTGTGTAACACTCACTCACAAACTTTTCAATTTGAGGGGAGGAC
       310                 330                 350
AGTGAATCTAAGATTGAAATTTATGAGTTTAATTAGACTAATTCCCATTTGATTTATTGG
       370                 390                 410
CTAGAAGTCAATTATTTGCATAGTGAGTCTTTTAACACACAGATTTGAGTTAAAGCTACT
       430                 450                 470
ACGTTCGTATTAACCCATAACATATACACCTTCTGTTCTAATTTCTTTGACACTTTTTGT
       490                 510                 530
TAGTTTGTTCCAAAAAGGACGGACATATTTGATATTTGAGAATACTTTACCTTAACCTTA
       550                 570                 590
ATAGAATTTTTTATGACATCACATATATTATGGAATATATACGACCATAATTTTCAAATA
       610                 630                 650
TCTTATAGTCGTACAAATATTATAGCATGTTTAATACCACAACTTTCAAATTCTTCTTTT
       670                 690                 710
CCTTAAAAACAAAATATGTCACATAAATTAAAATAGAGGAAGTATACTACATCAATCAGC
       730                 750                 770
CCCTAGTGGAGGGGACCTACTGTAAGTTTTTAAGTTTTCAAGAATTCAGTAATTGATTAG
       790                 810                 830
GAGCCCGTCTGGACATAAAAAAAAATTCCTTTTTTTCCAAAAAATGCCCACTAAATTTCT
       850                 870                 890

           intron <---------  A  Q  S  I  G  V  C  Y  G  M
AACACTATTTTGTAATTCTTATTGAGCAGGGGCTCAATCGATAGGTGTTTGCTATGGAAT
        910                 930                 950
 L  G  N  N  L  P  N  H  W  E  V  I  Q  L  Y  K  S  R  N  I
GCTAGGCAACAACTTGCCAAATCATTGGGAAGTTATACAGCTCTACAAGTCAAGAAACAT
        970                 990                1010
 G  R  L  R  L  Y  D  P  N  H  G  A  L  Q  A  L  K  G  S  N
AGGAAGACTGAGGCTTTATGATCCAAATCATGGAGCTTTACAAGCATTAAAAGGCTCAAA
       1030                1050                1070
 I  E  V  M  L  G  L  P  N  S  D  V  K  H  I  A  S  G  M  E
TATTGAAGTTATGTTAGGACTTCCCAATTCAGATGTGAAGCACATTGCTTCCGGAATGGA
       1090                1110                1130
 H  A  R  W  W  V  Q  K  N  V  K  D  F  W  P  D  V  K  I  K
ACATGCAAGATGGTGGGTACAGAAAAATGTTAAAGATTTCTGGCCAGATGTTAAGATTAA
       1150                1170                1190
 Y  I  A  V  G  N  E  I  S  P  V  T  G  T  S  Y  L  T  S  F
GTATATTGCTGTTGGGAATGAAATCAGCCCTGTCACTGGCACATCTTACCTAACCTCATT
       1210                1230                1250
 L  T  P  A  M  V  N  I  Y  K  A  I  G  E  A  G  L  G  N  N
TCTTACTCCTGCTATGGTAAATATTTACAAAGCAATTGGTGAAGCTGGTTTGGGAAACAA
       1270                1290                1310
```

FIG 4(vervolg)

```
 I  K  V  S  T  S  V  D  M  T  L  I  G  N  S  Y  P  P  S  Q
CATCAAGGTCTCAACTTCTGTAGACATGACCTTGATTGGAAACTCTTATCCACCATCACA
       1330                1350                1370
 G  S  F  R  N  D  A  R  W  F  V  D  A  I  V  G  F  L  R  D
GGGTTCGTTTAGGAACGATGCTAGGTGGTTTGTTGATGCCATTGTTGGCTTCTTAAGGGA
       1390                1410                1430
 T  R  A  P  L  L  V  N  I  Y  P  Y  F  S  Y  S  G  N  P  G
CACACGTGCACCTTTACTCGTTAACATTTACCCCTATTTCAGTTATTCTGGTAATCCAGG
       1450                1470                1490
 Q  I  S  L  P  Y  S  L  F  T  A  P  N  V  V  V  Q  D  G  S
CCAGATTTCTCTCCCCTATTCTCTTTTTACAGCACCAAATGTGGTGGTACAAGATGGTTC
       1510                1530                1550
 R  Q  Y  R  N  L  F  D  A  M  L  D  S  V  Y  A  A  L  E  R
CCGCCAATATAGGAACTTATTTGATGCAATGCTGGATTCTGTGTATGCTGCCCTCGAGCG
       1570                1590                1610
 S  G  G  A  S  V  G  I  V  V  S  E  S  G  W  P  S  A  G  A
ATCAGGAGGGGCATCTGTAGGGATTGTTGTGTCCGAGAGTGGCTGGCCATCTGCTGGTGC
       1630                1650                1670
 F  G  A  T  Y  D  N  A  A  T  Y  L  R  N  L  I  Q  H  A  K
ATTTGGAGCCACATATGACAATGCAGCAACTTACTTGAGGAACTTAATTCAACACGCTAA
       1690                1710                1730
 E  G  S  P  R  K  P  G  P  I  E  T  Y  I  F  A  M  F  D  E
AGAGGGTAGCCCAAGAAAGCCTGGACCTATTGAGACCTATATATTTGCCATGTTTGATGA
       1750                1770                1790
 N  N  K  N  P  E  L  E  K  H  F  G  L  F  S  P  N  K  Q  P
GAACAACAAGAACCCTGAACTGGAGAAACATTTTGGATTGTTTTCCCCCAACAAGCAGCC
       1810                1830                1850
 K  Y  N  I  N  F  G  V  S  G  G  V  W  D  S  S  V  E  T  N
CAAATATAATATCAACTTTGGGGTCTCTGGTGGAGTTTGGGACAGTTCAGTTGAAACTAA
       1870                1890                1910
 A  T  A  S  L  V  S  E  M  *
TGCTACTGCTTCTCTCGTAAGTGAGATGTGAGCTGATGAGACACTTGAAATCTCTTTACA
       1930                1950                1970
TACGTATTCCTTGGATGGAAAACCTAGTAAAAACAAGAGAAATTTTTTCTTTATGCAAGA
       1990                2010                2030
TACTAAATAACATTGCATGTCTCTGTAAGTCCTCATGGATTGTTATCCAGTGACGATGCA
       2050                2070                2090
ACTCTGAGTGGTTTTAAATTCCTTTTCTTTGTGATATTGGTAATTTGGCAAGAAACTTTC
       2110                2130                2150
TGTAAGTTTGTGAATTTCATGCATCATTAATTATACATCAGTTCCATGTTTGATCAGATT
       2170                2190                2210
GGGATTTGGTAACTTCAATGTTAGTATTATAATTAGTGTCTTTATCATTGACTATCAATT
       2230                2250                2270
AATCTTTATTTGGCAAGGCTTGATATATTTGAGTTACTCTTAGGTATTTGCAAGCAACTG
       2290                2310                2330
ATCTTTCTTTTATCCCGTTTCTGGCTTAAACCTCATTAGAAATATATTATAATGTCACCT
       2350                2370                2390
ACTCTGTGGTTTAAGACATTCCCTTACATTATAAGGTATTTCACGTCGTATCAGGTCGAA
       2410                2430                2450
AAAAATAATGGTACGCTCTTTCTTATCACAAATTTCTCTAACTTCTAGA
       2470                2490
```

FIG 5

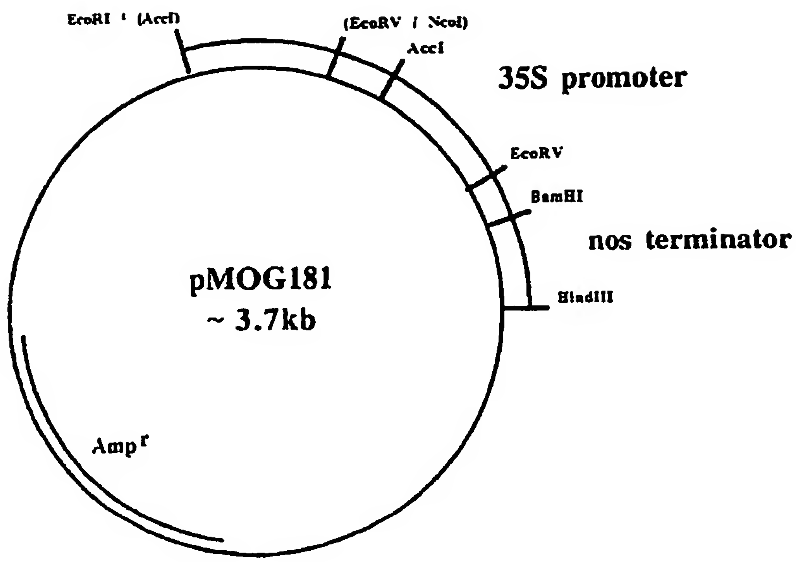

EcoRI / (AccI)
(EcoRV / NcoI)
AccI
35S promoter
EcoRV
BamHI
nos terminator
HindIII
pMOG181
~ 3.7kb
Amp r

FIG 6

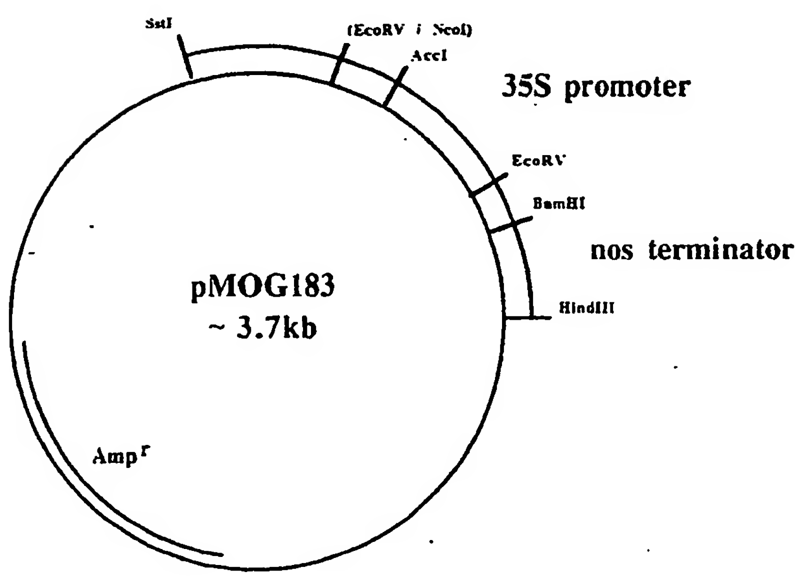

SstI
(EcoRV / NcoI)
AccI
35S promoter
EcoRV
BamHI
nos terminator
HindIII
pMOG183
~ 3.7kb
Amp r

FIG 7

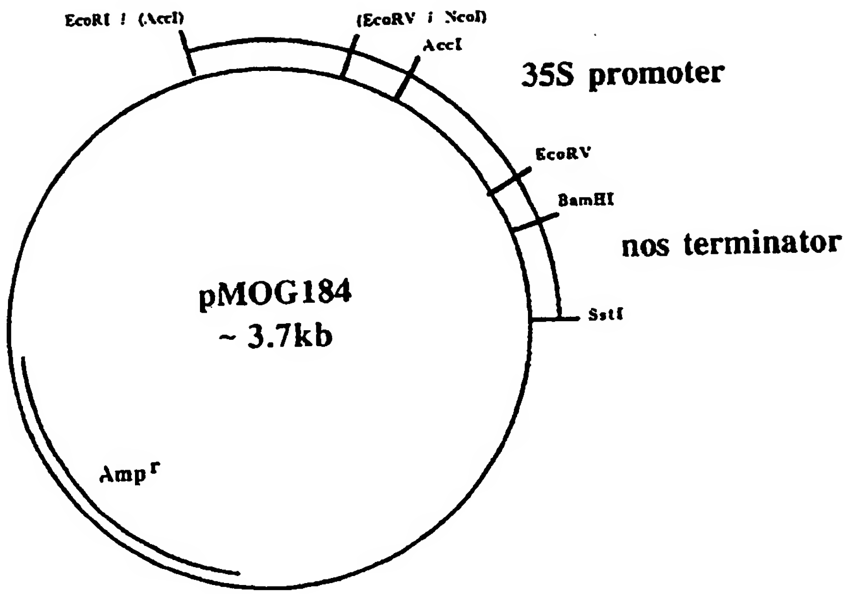

EcoRI / (AccI)
(EcoRV / NcoI)
AccI
35S promoter
EcoRV
BamHI
nos terminator
SstI
pMOG184
~ 3.7kb
Amp r

FIG 8

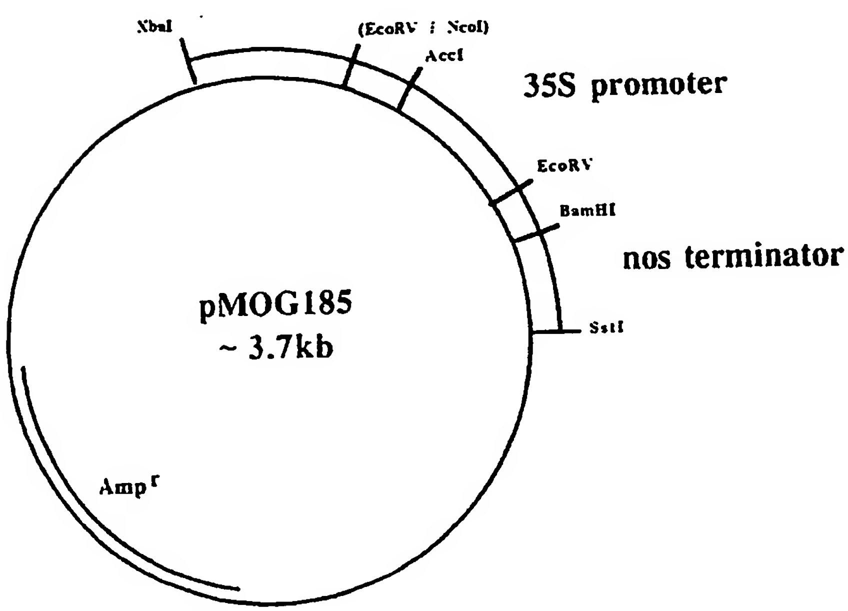
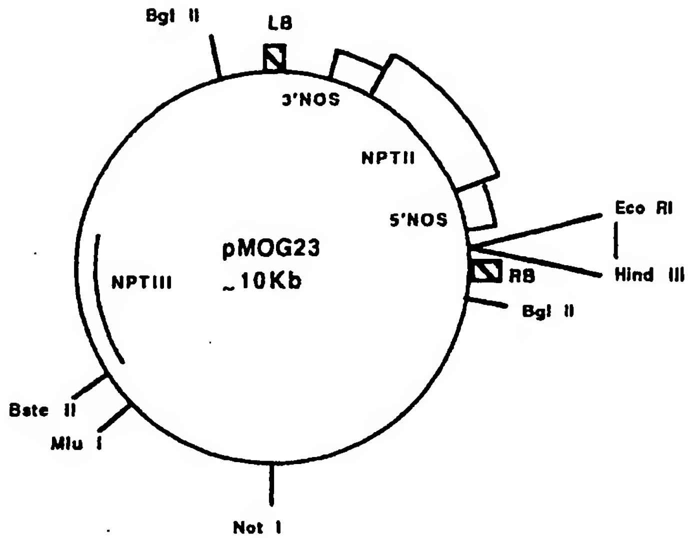

XbaI
(EcoRV / NcoI)
AccI
35S promoter
EcoRV
BamHI
nos terminator
SstI
pMOG185
~ 3.7kb
Amp r

FIG 9



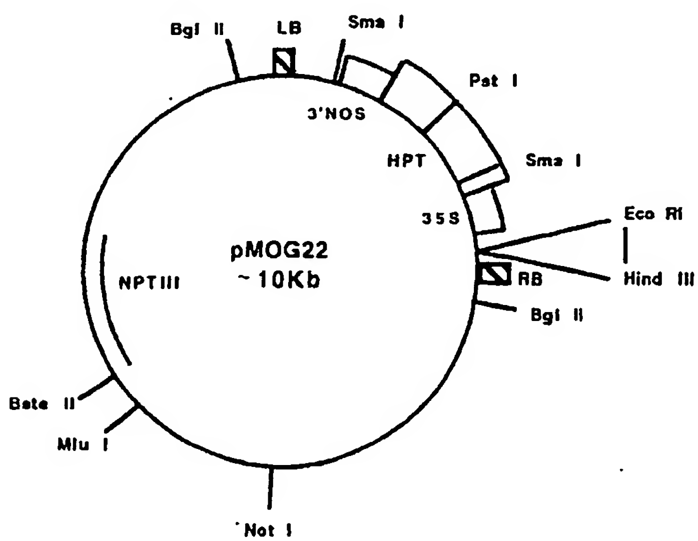
Polylinker sequence:

Eco RI Kpn I Sma I Bam HI Xba I Xho I Hind III

5' GGAATTCTGGTACCTCCCGGGAGGATCCATCTAGAGCTCGAGTAAGCTTC 3'

Sac I

FIG 10



Polylinker sequenc :

Eco RI Kpn I Sma I Bam HI Xba I Xho I Hind III

5' GGAATTCTGGTACCTCCCGGGAGGATCCATCTAGAGCTCGAGTAAGCTTC 3'

Sac I

FIG 11

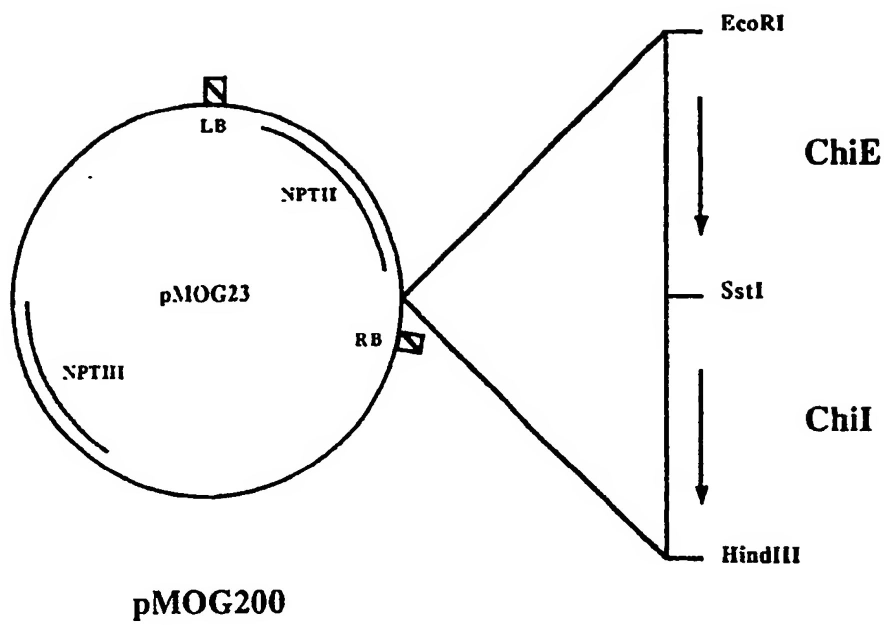
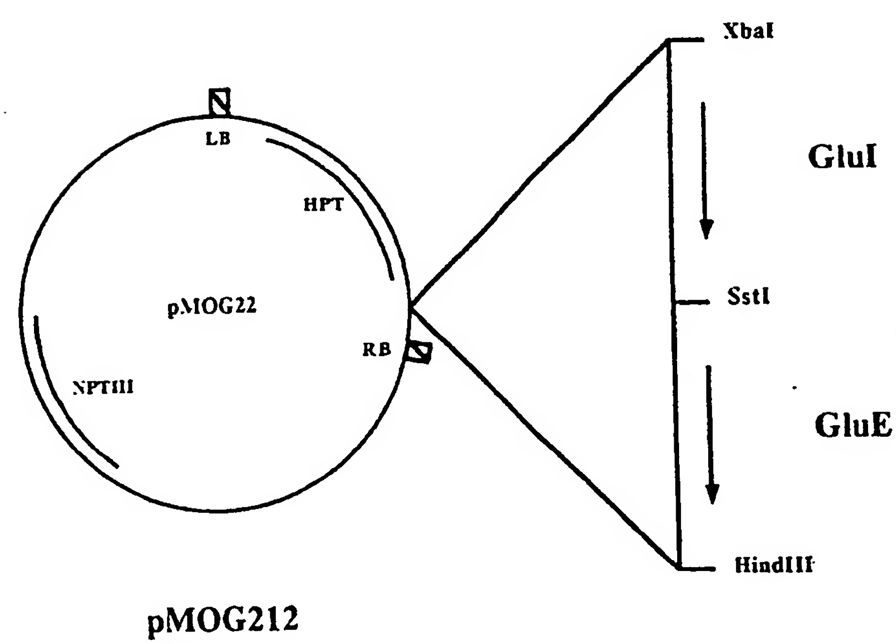

EcoRI
LB
ChiE
NPTII
pMOG23
SstI
RB
NPTIII
ChiI
HindIII
pMOG200

FIG 12


XbaI
LB
GluI
HPT
pMOG22
SstI
RB
NPTIII
GluE
HindIII
pMOG212

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 20 0191

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-0 292 435 (CIBA-GEIGY) * Page 14, line 62 - page 15, line 4; example 16 *	13,24	C 12 N 15/56 C 12 N 15/82 A 01 H 5/00 C 12 N 1/21 A 01 N 65/00
Y		1,2,5,6,9, 12,16,18, 19,27	
Y	BIOLOGICAL ABSTRACTS, BR36: 106155, AN=89-216941; T.V. SUSLOW et al.: "Effect of expression of bacterial chitinase on tobacco susceptibility to leaf brown spot alternaria-longipes", & PHYTOPATHOLOGY 78 (12 PART 1). 1988. P. 1556 * Abstract *	1,2,12,20, 27	
Y	EP-A-0 270 248 (ICI) * Page 3, lines 4-6; claims 9,10 *	2,5,6,9, 16,18,19, 20	
P,Y	THE PLANT CELL, vol. 2, December 1990, pages 1145-1155, American Society of Plant Physiologists; S.Y. BEDNAREK et al.: "A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco" * Abstract; page 1150, left-hand column, paragraph 2 - page 1151, left-hand column, paragraph 2; page 1152, left-hand column *	2-4,14, 15,21,22	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N A 01 N
P,Y	PLANT MOLECULAR BIOLOGY, vol. 15, 1990, pages 797-808, Kluwer Academic Publishers, BE; G. PAYNE et al.: "Evidence for a third structural class of beta-1,3-glucanase in tobacco" * Whole document particularly figure 7 *	2-4,14, 15,21,22	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	03 May 91	MADDOX A.D.

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same catagory
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention

E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 20 0191

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
O,P,X	J. CELL. BIOCHEM., suppl. 15A, page 9, abstract no. A 015; J.F. BOL et al.: "Expression and targeting of.tobacco proteins induced by virus infection" * Abstract *	1-26	
O,P,X	J. CELL. BIOCHEM., suppl. 15A, page 49, abstract no. A 135; P.J.M. VAN DEN ELZEN et al.: "Antifungal activity of chitinases expressed in transgenic tobacco" * Abstract *	1,6,12,13, 16,17,24, 25,26,27	
P,X	BIOLOGICAL ABSTRACTS, vol. 90, no. 6, abstract no. 62613, Biological Abstract Inc., Philadelpia, PA, US; H.J.M. LINTHORST et al.: "Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco", & MOL. PLANT-MICROBE INTERACT 3(4): 252-258, 1990 * Abstract *	1,13,24	
P,X	WO-A-9 007 001 (DU-PONT) * Whole document *	1,12,13, 24,27	
P,X	EP-A-0 392 225 (CIBA-GEIGY) * Page 45, line 35 - page 49, line 7; page 58, lines 15-30; page 71, lines 30-56 *	1,12,13, 20,24	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
P,X	WO-A-9 009 436 (CARLSBERG) * Pages 15,16 *	20	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	03 May 91	MADDOX A.D.

CATEGORY F CITED DOCUMENTS
X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same catagory
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention

E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 20 0191

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 332 104 (CIBA-GEIGY) * Examples 24,32D,E,F,G * – – –	1-27	
A	DERWENT WPIL DATABASE, accession no. 89-337040, Derwent Publications Ltd, London, GB; & JP-A-1 252 292 (MITSUBISHI KASEI CORP.) * Abstract * – – –	20,27	
A	PLANT PHYSIOL., vol. 91, September 1989, pages 130-135; P. LUND et al.: "Bacterial chitinase is modified and secreted in transgenic tobacco" * Whole document * – – –	1-27	
A	THE EMBO JOURNAL, vol. 5, no. 9, 1986, pages 2057-2061, IRL Press Ltd, London, GB; R.A.M. HOOFT VAN HUIJSDUIJNEN et al.: "cDNA cloning of six nRNAs induced by TMV infection of tobacco and a characterization of their translation products" * Page 2061, left-hand column * – – –	1-27	
E	EP-A-0 418 695 (CIBA-GEIGY) * Pages 30-38 * – – – – –	1,12,13, 20,24	TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	03 May 91	MADDOX A.D.

CATEGORY OF CITED DOCUMENTS
X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same catagory
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention

E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document